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(54) Title: GENES WHICH INFLUENCE *PICHIA* PROTEOLYTIC ACTIVITY, AND USES THEREFOR

(57) Abstract

The isolation and characterization of genes involved in proteolytic processing in species of the genus *Pichia* is described. The availability of such genes has enabled the generation of strains of *Pichia* that are deficient in proteolytic activity and useful as hosts for the expression of proteolytically sensitive recombinant products. The isolation and characterization of additional genes from species of the genus *Pichia* is also described, as well as uses therefor.

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GENES WHICH INFLUENCE *PICHIA* PROTEOLYTIC ACTIVITY, AND USES THEREFOR

This invention relates to recombinant DNA technology. In a particular aspect, the present invention relates to yeast strains produced employing recombinant techniques, and DNA encoding proteins involved in proteolytic processing, as well as auxotrophic marker proteins. In another aspect, the present invention relates to methods of producing recombinant products, especially recombinant products which are susceptible to proteolytic degradation.

BACKGROUND OF THE INVENTION

Strains of the genus *Pichia* have been developed as an efficient expression system for the production of recombinant products. Unfortunately, however, some protein products which are desirably produced by recombinant means (e.g., IGF-1, EGF, GRF, and the like) are susceptible to degradation by proteases produced by the host organism. In such cases, even if high levels of the desired product are expressed, reduced product recoveries are sometimes realized due to degradation of the product in the presence of certain of the host strain's proteolytic enzymes. Product recovery is further complicated by the presence of various proteolysis degradation products.

It would be desirable, in view of the excellent performance of the *Pichia*-based expression system for the production of many recombinant products, to reduce or eliminate certain proteolytic activities of *Pichia*. This would reduce the likelihood of degradation of protease-sensitive products when produced in recombinant

Pichia hosts. Reduced likelihood of degradation would result in an enhanced ability to express and recover such products in substantially intact form.

Various techniques can be applied in an effort to reduce or eliminate the problem of proteolytic degradation of recombinantly produced products. For example, one could modify the conditions under which recombinant strains are grown so as to inhibit protease activity. This could be accomplished, for example, by adjusting the pH of the medium sufficiently to inhibit the action of various proteases. This approach, however, may affect the ability of the host organism to express certain recombinant products (as well as the stability of the resulting product, once expressed). Moreover, this approach is limited only to its effect on extracellular proteolysis.

Alternatively, one could attempt to modify or eliminate some or all of the host organism's processing enzymes which are responsible for the proteolytic activity which degrades recombinantly produced, proteolytically sensitive products. Proteolytic processes in eukaryotic organisms are, however, quite complicated and involved. Thus, it is not possible to predict if elimination and/or modification of one or more of the enzyme(s) that are involved in proteolytic processing pathways will have an impact on the viability of the host cells, and/or the stability of the recombinantly produced products.

Some of the proteolytic activities of the yeast *Saccharomyces cerevisiae* have been characterized. Proteinase A, for example, is encoded by the *S. cerevisiae PEP4* gene. Proteinase A is a vacuolar, aspartyl protease capable of self-activation, as well as subsequent activation of additional vacuolar proteases, such as carboxypeptidase Y, and proteinase B. Although

carboxypeptidase Y appears to be completely inactive prior to proteinase A-mediated proteolytic processing of the enzyme, proteinase B (encoded by the PRB-1 gene of *S. cerevisiae*) reportedly is approximately 50% bioactive in its precursor form, the form that exists prior to proteinase A-mediated processing of the enzyme.

S. cerevisiae and filamentous fungi deficient in proteolytic activity have been used for the recombinant expression of heterologous peptides. These organisms, however, differ substantially from the methylotrophic yeast, *Pichia*. There are numerous metabolic and physiological differences between *Saccharomyces*, *Aspergillus*, and *Pichia*, so that the proteolytic processing systems of these various organisms are not necessarily similar. Indeed, very little is presently known regarding the types of proteolytic activities present in *Pichia*.

In addition, unlike *Saccharomyces* or *Aspergillus*, *Pichia* cells used for the recombinant expression of heterologous peptides are typically grown to high cell density, which has been made possible, at least in part, by selection of strains which minimize the occurrence of foaming during the fermentation process. Selection of such cells is accomplished by selecting for cells which produce large amounts of endo- and exo-proteases, which reduce foaming by reducing the size of proteins secreted into the medium. Furthermore, while growth at high cell density the production of heterologous peptides in high yields, growth at high cell density also provides for a relatively high level of vacuolar proteases in the fermentation medium. The high cell density is accompanied by the release of substantial quantities of cellular material into the media, including vacuolar proteases, since ~1% of cells typically undergo lysis during yeast fermentation. Therefore, during the

production of heterologous peptides in a high cell density process, some of the secreted, heterologous peptides produced by *Pichia* could be subjected to substantial proteolysis.

Therefore, it is an object herein, to provide protease deficient strains of *Pichia* and to provide means for generating such strains. It is also an object to use the protease deficient strains for expression of heterologous proteins.

SUMMARY OF THE INVENTION

In accordance with the present invention, we have isolated and characterized genes involved in proteolytic processes of species of the genus *Pichia*. The availability of such genes provides a means to generate strains of *Pichia* which are deficient in proteolytic activity and which are useful as hosts for the expression of proteolytically sensitive products.

The strains of *Pichia* which have been modified so as to be defective in proteolytic activity, compared to wild-type *Pichia* cells, are excellent hosts for the expression of recombinant constructs encoding proteolytically sensitive products. The advantage of high levels of recombinant product expression using the *Pichia* expression system, coupled with the low level of proteolytic activity in the protease-deficient host cells provided herein, provides a highly efficient expression system for the production of proteolytically sensitive products.

In accordance with another embodiment, a gene that encodes the *Pichia* orotidine-5'-phosphate decarboxylase protein (the *URA3* gene) is provided. The availability of this gene, in combination with strains of *Pichia* which are Ura$^-$, provides a selection system for use in producing recombinant strains of *Pichia* which are deficient in proteolytic activity. Such Ura$^-$ strains are also useful

as hosts for transformation with recombinant DNA constructs, which are then used for the recombinant expression of a variety of heterologous products.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a restriction map of a *Pichia pastoris* gene which influences the carboxypeptidase Y activity of *Pichia*.

Figure 2 is a restriction map of plasmid pEP202.

Figure 3 is a restriction map of plasmid pEP205.

Figure 4 is a restriction map of plasmid pEP301.

Figure 5 is a restriction map of plasmid pDR401.

Figure 6 is a restriction map of plasmid pPU201.

Figure 7 is a restriction map of plasmid pPU202.

Figure 8 is a restriction map of plasmid pPU203.

Figure 9 is a restriction map of plasmid pPU205.

Figure 10 is a restriction map of plasmid pPU206.

Figure 11 is a restriction map of plasmid pDR421.

Figure 12 is a restriction map of the *Pichia pastoris* orotidine-5'-phosphate decarboxylase gene (i.e., the *URA3* gene).

Figure 13 summarizes the steps employed in the construction of pDR601 and pDR602.

Figure 14 is a restriction map of plasmid pDR601.

Figure 15 is a restriction map of plasmid pDR602.

Figure 16 is a restriction map of plasmid pDL521.

Figure 17 is a restriction map of a portion of the *Pichia pastoris* proteinase B gene.

Figure 18 is a restriction map of plasmid pDR911.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided an isolated DNA fragment obtained from a strain of the genus *Pichia* which comprises a gene encoding a protein which, directly or indirectly, influences the proteolytic activity of said strain.

In accordance with another embodiment of the present invention, there is provided a method of producing modified strain(s) of the genus *Pichia* which are deficient in proteolytic activity, relative to host strain(s) of the same species which are not so modified, said method comprising:

contacting said host strain(s) with a modified form of the above-described gene, wherein said modification renders the gene incapable of producing functional product, or alters the ability of the gene product to influence proteolytic activity, wherein said contacting is carried out under conditions suitable for the site-directed integration of said modified form of the above-described gene into the genome of said host strain(s), wherein said site-directed integration occurs at the specific locus of said gene which encodes said protein which influences proteolytic activity.

In accordance with yet another embodiment of the present invention, there are provided strains of the genus *Pichia* which are deficient in proteolytic activity. Such strains can be produced in a variety of ways, with the above-described method being the presently preferred way of producing such strains.

In accordance with still another embodiment of the present invention, there is provided a method for the expression of proteolytically sensitive recombinant product(s), said method comprising expressing said proteolytically sensitive product(s) in the above-described *Pichia* cells which are deficient in proteolytic activity.

In accordance with a further embodiment of the present invention, there is provided an isolated DNA fragment obtained from a species of the genus *Pichia* which comprises the orotidine-5'-phosphate decarboxylase gene.

In accordance with a still further embodiment of the present invention, there is provided a yeast cell of the genus *Pichia* as a host capable of being transformed with recombinant DNA material, wherein said host is defective in the orotidine-5'-phosphate decarboxylase gene.

As employed herein, the term "proteolytic activity" refers to any one or more of the enzyme activities displayed by enzymes involved in the proteolytic pathway. Proteolytic activities include proteinase A activity, proteinase B activity, carboxypeptidase Y activity, carboxypeptidase S activity, aminopeptidase C activity, dipeptidyl aminopeptidase activity, proteinase D activity, proteinase E activity, and the like.

As used herein, a gene encoding a protein which, directly or indirectly, influences the proteolytic activity of a yeast strain, includes genes that encode proteinases or that encode proteins that act on proteinases. As used herein, a protein that acts on a protein refer to proteins that alter or modulate the activity of a proteinase. Thus, for example, a protein that directly influences proteolytic activity is a protein that encodes a proteinase, and a protein that indirectly influences proteolytic activity is a protein that activates or increases the activity of a protein by proteolytic processing. *Saccharomyces cerevisiae* proteinase is an example of a protein that directly and indirectly influences the proteolytic activity of *Saccharomyces cerevisiae*.

In accordance with one embodiment of the present invention, the *Pichia* gene that encodes a protein which, directly or indirectly, influences at least the carboxypeptidase Y activity of strains of the genus *Pichia* has been identified and isolated from a species of the genus *Pichia*. This gene is referred to herein, for convenience, as the *Pichia PEP4* gene, based on the

existence of some similarity between this gene and the *S. cerevisiae PEP4* gene. It should be recognized, however, that the nucleotide sequences of the *Pichia* gene and the *Saccharomyces* gene differ substantially. The *Pichia PEP4* gene is characterized by the restriction map set forth in Figure 1 of the drawings. A fragment containing sequences encoding this gene can be readily obtained for easy handling from a variety of sources. One such source is the approximately 10.6 kbp EcoRI fragment of plasmid pEP202 (see Figure 2), or alternatively, the approximately 2.7 kbp EcoRI-SacI fragment of plasmid pEP301 (see Figure 4).

DNA encoding the proteinase A gene is also provided. The proteinase A gene of the present invention can be further characterized by reference to the amino acid sequence set forth in Sequence ID No. 2. DNA having any nucleic acid sequence which encodes substantially the same amino acid sequence as set forth in Sequence ID No. 2 or that has sufficient homology to be useful for disruption of homologous genes can be employed in the practice of the present invention. An exemplary nucleic acid sequence which encodes the above-described amino acid sequence is set forth in Sequence ID No. 1.

The *Pichia* gene that encodes a protein which, directly or indirectly, influences the proteolytic activity of strains of the genus *Pichia* can be modified in a variety of ways, so as to render the gene incapable of producing functional product, or so as to alter the ability of the gene product to influence the proteolytic activity of said *Pichia* strain(s). Those of skill in the art recognize that there are many methods for the modification of the above-described gene. For example, the coding sequence can be mutated to modify the amino acid sequence of the protein encoded by the gene. Alternatively, various portions of the coding sequence

can be deleted from the gene. The deletion need only be sufficient to render the expressed product (if it is still capable of being expressed) non-functional. Thus, a deletion of even one nucleotide, by throwing the remaining coding sequence out of reading frame, can render a product, if still capable of expression, non-functional. Of course, larger deletions can result in a complete lack of expression of product, or can cause a substantially modified product to be expressed, and such a product is likely to have very different proteolytic properties, if any, relative to product produced by intact gene. As yet another alternative, additional sequences can be inserted into the coding sequence to disrupt the reading frame of the gene of interest, which would cause a dramatically altered product to be expressed, or a complete lack of expression of the product.

A particularly convenient method for the modification of the *Pichia* gene that encodes a protein which, directly or indirectly, influences the proteolytic activity of strains of the genus *Pichia* is to insert an auxotrophic marker gene into said *Pichia* gene, thereby disrupting the *Pichia* gene. Such auxotrophic marker genes can be selected from the *Pichia* or *Saccharomyces HIS4* gene, the *Pichia* or *Saccharomyces ARG4* genes, the *Pichia* or *Saccharomyces URA3* genes, and the like.

Strains of *Pichia* deficient in proteolytic activity can be prepared in a variety of ways. The presently preferred method involves modifying, in a suitable host, genes of the present invention (which genes, in their unmodified form, encode a product which, directly or indirectly, affects the proteolytic activity of strains of the genus *Pichia*). Alternatively, host strains can be subjected to random (i.e., non-selective) mutagenesis,

then screened to select for mutants which are deficient in proteolytic activity.

When proteolytically deficient strains are produced by modifying the gene of the invention in a host, such modifying is carried out, for example, by introducing a modified gene under transformation conditions suitable for the site-directed integration of the modified gene into the genome of the host at the specific locus of such gene which encodes a protein which influences proteolytic activity (i.e., the target gene). Integration will replace or alter the host's endogenous gene. A convenient means to introduce the modified gene into the target locus of a yeast host is to include the modified gene in a linear DNA fragment having ends homologous to two separate portions of the intact gene within the host. This will direct, upon transformation, homologous recombination occur at the specific locus of the gene whose expression product influences proteolytic activity.

When *Pichia* strains deficient in proteolytic activity are prepared by the preferred method described above (i.e., by introducing a modified gene of the invention into a suitable host by site-directed integration at the specific locus of the gene whose expression product influences proteolytic activity, thereby replacing all or a portion of the endogenous gene with all or a portion of the modified gene), the endogenous gene is said to be disrupted.

As used herein, the term gene "disruption" refers to any manipulation of the target locus that ultimately results in the presence of a gene that does not yield a functional product, or that yields a product with altered function. Disruption can, therefore, result from the presence of added sequence (e.g., by the introduction of auxotrophic marker, or by the introduction of any sequence which causes a shift in the reading frame), the

loss of nucleotides from the target gene (e.g., by deletion), or other mutations of the target gene. For the preferred method of preparing *Pichia* strains deficient in proteolytic activity, gene disruption is achieved by gene addition, gene replacement, or a combination of addition and replacement referred to herein as "pop-in-pop-out". In gene replacement, the endogenous target gene is physically removed from the target locus, and replaced with the modified gene. This is accomplished by transforming the host with a linear fragment having ends which are homologous to the 5' and 3' ends of the target gene, respectively. Gene addition involves adding the transforming DNA to the endogenous target gene. Depending on the manner in which the modified gene of the transforming DNA was altered, gene addition can result in the presence of either two non-functional copies of the target gene, or one functional and one non-functional copy of the target gene. Each of the two copies consists of a portion of the endogenous gene, and a portion of the transforming DNA. If a functional copy of the target gene remains after gene addition, it can then be removed by homologous recombination between the two copies of the target gene. The combination process of gene addition followed by homologous recombination constitutes the pop-in-pop-out process.

Methods of transforming yeast of the genus *Pichia*, as well as methods applicable for culturing such yeast cells, are known generally in the art. Constructs containing the above-described modified gene are transformed into *Pichia* cells either by the spheroplast technique, described by Cregg et al., in Mol. Cell. Biol. 5:3376 (1985) and U.S. 4,879,231, or by the whole-cell lithium chloride yeast transformation system [Ito et al., Agric. Biol. Chem. 48:341 (1984)], with modification

necessary for adaptation to *Pichia* [See European Patent Application No. 312,934; also available as U.S. Pat. No. 4,929,535]. The whole-cell lithium chloride method is frequently more convenient in that it does not require the generation and maintenance of spheroplasts. For the purpose of the present invention, the spheroplast method is preferred because the spheroplast method is generally a more efficient means of transformation.

Those of skill in the art recognize that host *Pichia* strains for transformation with the above-described modified gene can be wild-type *Pichia* cells, which upon transformation with a defective gene from the proteolytic pathway, could be screened for reduced proteolytic activity. The host strains employed can have one or more defects therein, to assist in the identification and selection of desired transformants.

Preferred hosts employed for transformation with a modified form of the gene which encodes a protein which, directly or indirectly, influences the proteolytic activity of strains of *Pichia*, is a strain which is defective in at least one auxotrophic marker gene. The use of such host organisms is preferred because simultaneous transformation of such a host with the modified form of the invention gene and an auxotrophic marker gene enables rapid selection of strains which have incorporated the transforming DNA, and thus, should have a disrupted form of the gene which encodes a protein which directly or indirectly influences the proteolytic activity of the host.

Exemplary auxotrophic marker genes useful in the practice of the present invention (i.e., marker genes that are defective in the preferred host strains employed herein) include the histidinol dehydrogenase gene, the argininosuccinate lyase gene, or the orotidine-5'-phosphate decarboxylase gene, and the like.

When employing such host strains in the transformation of *Pichia*, the above-described modified gene, included on a linear DNA fragment, is preferably associated with an intact form of the auxotrophic marker gene for which the host strain is defective, e.g., the auxotrophic marker gene either is contained within the modified gene, or is located 5' or 3' of the modified gene on the transforming linear DNA fragment. Exemplary host strains contemplated for use in the practice of the present invention include the *HIS4*-defective *Pichia* strain, GS115 (ATCC 20864), the *ARG4*-defective *Pichia* strain, GS190, the *HIS4/URA3*-defective *Pichia* strain, GS4-2, the *HIS4/ARG4*-defective *Pichia* strain PPF1 (NRRL Y-18017; see U.S. 4,812,405), and the like. An exemplary fragment of DNA which contains the above-described modified gene having inserted therein a functional gene encoding histidinol dehydrogenase can be obtained from the approximately 5.3 kbp SacI-EcoRI fragment of plasmid pDR401. Another exemplary fragment of DNA which contains a modified form of the above-described gene (located 5' of a functional gene encoding orotidine-5'-phosphate decarboxylase) can be obtained from the approximately 5.0 kbp BglII fragment of plasmid pDR421.

A particularly advantageous application of the *Pichia* strains that are deficient in proteolytic activity is for the expression of proteolytically sensitive recombinant products, such as, for example, epidermal growth factor (EGF), growth hormone releasing factor (GRF), insulin-like growth factor-1 (IGF-1), and the like. When expressed in recombinant *Pichia* strains, which are deficient in proteolytic activity, the resulting recombinant product is subjected to a reduced level of proteolytic activity, due to modifications in the proteolysis apparatus of the host organism.

Proteolytically deficient *Pichia* expression systems for the production of proteolytically sensitive products can be generated in a variety of ways. For example, *Pichia* host strains can be rendered proteolytically deficient, as described hereinabove, and then further transformed with DNA encoding a heterologous protein of interest (especially a proteolytically sensitive protein). Alternatively, a recombinant *Pichia* strain already bearing DNA encoding a heterologous protein of interest can thereafter be rendered proteolytically deficient, for example, as described hereinabove. As yet another alternative, a *Pichia* strain could be co-transformed with the above described modified gene and a DNA encoding a heterologous, proteolytically sensitive protein of interest.

The use of strains of the genus *Pichia* as host strains in the recombinant expression of peptide products has previously been described in great detail. The presently preferred yeast species for use in the practice of the present invention is *Pichia pastoris*, a known industrial yeast strain that is capable of efficiently utilizing methanol as the sole carbon and energy source.

There are a number of methanol-responsive genes in methylotrophic yeast, the expression of each being controlled by methanol-responsive regulatory regions (also referred to as promoters). Any of such methanol-responsive promoters are suitable for use in the practice of the present invention. Examples of specific regulatory regions include the promoter for the primary alcohol oxidase gene from *Pichia pastoris* AOX1, the promoter for the secondary alcohol oxidase gene from *P. pastoris AOX2* (*P. pastoris* is known to contain two functional alcohol oxidase genes: alcohol oxidase I (AOX1) and alcohol oxidase II (*AOX2*); the coding portions

of the two AOX genes are closely homologous at both the DNA and the predicted amino acid sequence levels and share common restriction sites; the proteins expressed from the two genes have similar enzymatic properties but the promoter of the AOX1 gene is more efficient and gene products are frequently more highly expressed therefrom), the promoter for the dihydroxyacetone synthase gene from *P. pastoris* (*DAS*), the promoter for the P40 gene from *P. pastoris*, the promoter for the catalase gene from *P. pastoris*, the promoter for the formaldehyde dehydrogenase gene from *P. pastoris*, the promoter for the formate dehydrogenase gene from *P. pastoris*, and the like.

The presently preferred promoter region for regulating expression of a gene encoding a proteolytically sensitive product, in *P. pastoris* hosts, is the promoter of the methanol-regulated primary alcohol oxidase gene of *P. pastoris*. The AOX1 gene, including its promoter, has been isolated and thoroughly characterized; see Ellis et al., Mol. Cell. Biol. 5: 1111 (1985) and U.S. Patent No. 4,855,231.

The presently preferred expression cassette used in transforming *Pichia* cells for the generation of recombinant protein-expressing strains comprises, in the reading frame direction of transcription, the following DNA sequences:

(i) a promoter region of a methanol-responsive gene of a methylotrophic yeast,

(ii) a DNA sequence encoding a polypeptide consisting essentially of:

(a) an optional secretion signal sequence, and

(b) a heterologous protein of interest; and

(iii) a transcription terminator functional in a methylotrophic yeast;

wherein said DNA sequences are operationally associated with one another for transcription of the sequences

encoding said polypeptide. DNA sequences encoding a secretion signal sequence which are optionally contained in expression vectors used in the practice of the present invention include the DNA encoding the native secretion signal sequence associated with the proteolytically sensitive product, the DNA encoding the *S. cerevisiae* α-mating factor (αMF) leader sequence, (including a DNA sequence encoding the processing site, lys-arg), and signal sequences that function as such in methylotrophic yeast cells, such as the bovine lysozyme C signal sequence.

The transcription terminator functional in a methylotrophic yeast used in accordance with the present invention has either (a) a subsegment which provides a polyadenylation signal and polyadenylation site in the transcript, and/or (b) a subsegment which provides a transcription termination signal for transcription from the promoter used in the expression cassette. The term "expression cassette" as used herein, and throughout the specification and claims, refers to a DNA sequence which includes sequences functional for the expression process. The entire transcription terminator is taken from a protein-encoding gene, which may be the same or different from the gene which is the source of the promoter.

In the DNA constructs of the present invention, used to transform hosts for recombinant expression of proteolytically sensitive products, the segments of the expression cassette(s) are said to be "operationally associated" with one another. The DNA sequence encoding proteolytically sensitive products is positioned and oriented functionally with respect to the promoter, the secretion signal sequence, if employed, and the transcription terminator. Thus, the polypeptide-encoding segment is transcribed, under regulation of the promoter region, into a transcript capable of providing, upon

translation, the desired polypeptide. Appropriate reading frame positioning and orientation of the various segments of the expression cassette are within the knowledge of persons of ordinary skill in the art; further details are given in the Examples.

For the practice of the present invention it is preferred that hosts for the recombinant expression of proteolytically sensitive products be transformed with multiple copies of the above-described expression cassettes contained on one DNA fragment, preferably in a head-to-tail orientation.

In addition, when DNA constructs according to the invention are used to transform hosts for the recombinant expression of proteolytically sensitive products by site-directed integration, the expression cassette-containing construct is a linear DNA fragment that is directed to the desired locus of the host to effect integration of the DNA fragment therein. One-step gene integrations are usually successful if the DNA to be introduced has as little as 0.2 kb homology with the fragment locus of the target gene; it is however, preferable to maximize the degree of homology for efficiency.

The DNA constructs used according to the invention to transform hosts for the recombinant expression of proteolytically sensitive products optionally further comprise a selectable marker gene, in addition to one or more expression cassettes. For this purpose, any selectable marker gene functional in methylotrophic yeast may be employed, i.e., any gene which confers a phenotype upon methylotrophic yeast cells, thereby allowing them to be identified and selectively grown from among a vast majority of untransformed cells. Suitable selectable marker genes include, for example, selectable marker systems composed of an auxotrophic mutant *P. pastoris*

host strain and a wild-type biosynthetic gene which complements the host's defect. For transformation of *HIS4*⁻ *P. pastoris* strains, for example, the *S. cerevisiae* or *P. pastoris HIS4* gene may be employed, or for transformation of *ARG4*⁻ mutant *P. pastoris* strains, the *S. cerevisiae ARG4* gene or the *P. pastoris ARG4* gene may be employed, or for transformation of *URA3*⁻ mutant *P. pastoris* strains, the *S. cerevisiae URA3* gene or the *P. pastoris URA3* gene may be employed.

In addition, DNA constructs used to transform hosts for the recombinant expression of proteolytically sensitive products according to this aspect of the invention optionally further comprise selectable marker genes which are functional in bacteria. Thus, any gene can be used which confers a phenotype on bacteria that allows transformed bacterial cells to be identified and selectively grown from among a vast majority of untransformed cells. This additional selectable marker enables DNA of the invention to be transformed into bacteria such as *E. coli* for amplification. Suitable selectable marker genes include the ampicillin resistance gene (Amp^r), tetracycline resistance gene (Tc^r), and the like.

When it is contemplated to pass DNA of the invention though bacterial cells, it is desirable to include in the DNA construct a bacterial origin of replication, to ensure the maintenance of the invention DNA from generation to generation of the bacteria. Exemplary bacterial origins of replication include the f1-ori, colisin, col El, and the like.

The term "expression vector", as employed herein, is intended to include vectors capable of expressing DNA sequences contained therein, where such sequences are in operational association with other sequences capable of effecting their expression, i.e., promoter sequences. In

general, expression vectors usually used in recombinant DNA technology are often in the form of "plasmids", i.e., circular, double-stranded DNA loops, which in their vector form are not bound to the chromosome. In the present specification the terms "vector" and "plasmid" are used interchangeably. However, the invention is intended to include other forms of expression vectors as well, which function equivalently.

Methods of transforming yeast of the genus *Pichia*, as well as methods applicable for culturing such yeast cells, are known generally in the art.

According to the invention, constructs containing the above-described modified gene and/or expression cassettes encoding the production of heterologous, proteolytically sensitive products are transformed into *Pichia* cells either by the spheroplast technique, or by the whole-cell lithium chloride yeast transformation system, as described above.

Transformed strains, which are of the desired phenotype and genotype, are grown in fermentors in either batch or continuous mode. For the large-scale production of recombinant DNA-based products in methylotrophic yeast, a three-stage, high cell-density fermentation system is the presently preferred fermentation protocol employed. In the first, or growth stage, expression hosts are cultured in defined minimal medium with an excess of a non-inducing carbon source (e.g., glycerol). When grown on such carbon sources, heterologous gene expression is completely repressed, which allows the generation of cell mass in the absence of heterologous protein expression. It is presently preferred, during this growth stage, that the pH of the medium be maintained at about 5, because the *P. pastoris* cells generally prefer a pH of about 5 for optimal growth. Next, a short period of non-inducing carbon source

limitation growth is allowed to further increase cell mass and derepress the methanol-responsive promoter. The pH of the medium during this limitation growth period is maintained at an appropriate pH value (the actual pH employed is a function of the particular host strain used for expression and the specific product being expressed).

Subsequent to the period of growth under limiting conditions, methanol is added in the fermentor either on a continuous basis, with concurrent removal of product via the broth; or on a batch-wise basis wherein methanol is added at such a rate that the methanol content of the broth is maintained at a low level (referred to herein as "methanol excess fed-batch mode"). The addition of methanol induces the expression of the gene driven by a methanol-responsive promoter. This third stage is referred to as the production stage, because it is at this stage that the majority of the recombinant product is expressed. The pH of the medium during the production stage is maintained at an appropriate pH value (the actual pH employed is a function of the particular host strain used for expression and the specific product being expressed).

The term "culture" means a propagation of cells in a medium conducive to their growth, and all sub-cultures thereof. The term "subculture" refers to a culture of cells grown from cells of another culture (source culture), or any subculture of the source culture, regardless of the number of subculturing steps that have been performed between the subculture of interest and the source culture.

According to a preferred embodiment of the present invention, the heterologous protein expression system used for the production of proteolytically sensitive products utilizes the promoter derived from the methanol-regulated *AOX1* gene of *P. pastoris*, which is very

efficient and tightly regulated. This gene can be the source of the transcription terminator as well. The presently preferred expression cassette comprises, operationally associated with one another, the *P. pastoris* AOX1 promoter, optional DNA encoding a secretion signal sequence, a DNA sequence encoding a proteolytically sensitive product (e.g., mature IGF-1, EGF, GRF, and the like), and a transcription terminator derived from the *P. pastoris* AOX1 gene. Preferably, two or more of such expression cassettes are contained on one DNA fragment, in head-to-tail orientation, to yield multiple expression cassettes on a single contiguous DNA fragment.

The presently preferred host cells to be transformed with multiple expression cassettes are *P. pastoris* cells having at least one mutation that can be complemented with a marker gene present on a transforming DNA fragment. Preferably *HIS4*$^-$ (GS115) or *ARG4*$^-$ (GS190) single auxotrophic mutant *P. pastoris* strains are employed, or *HIS4*$^-$/*URA3*$^-$ (GS4-2) or *HIS4*$^-$/*ARG4*$^-$ (PPF1) double auxotrophic mutant *P. pastoris* strains are employed.

The fragment containing one or more expression cassette(s) is inserted into a plasmid containing a marker gene complementing a metabolic defect in the host, and optionally containing additional sequences such as bacterial marker genes, yeast DNA sequences which direct vector integration, and the like.

In accordance with a specific embodiment of the present invention, there is provided an isolated DNA fragment obtained from a species of the genus *Pichia* which comprises the orotidine-5'-phosphate decarboxylase gene. The orotidine-5'-phosphate decarboxylase gene is frequently referred to as *URA3*. It can be used, for example, to complement *URA3*-deficient strains. Another use for the novel gene is the ability to target DNA into

a specific locus of the *Pichia* genome (i.e., into the *URA3* locus). This novel gene can be characterized by reference to the restriction map shown in Figure 12. Alternatively, this novel gene can be characterized as encoding a protein having substantially the same amino acid sequence as set forth in Sequence ID No. 4. While those of skill in the art recognize that the above-referenced amino acid sequence can be encoded by a variety of nucleotide sequences, a presently preferred nucleotide sequence encoding the above-referenced amino acid sequence is substantially the same as that set forth in Sequence ID No. 3.

In accordance with another specific embodiment of the present invention, there are provided yeast cells of the genus *Pichia* as a host capable of being transformed with recombinant DNA material, wherein the host is defective in the orotidine-5'- phosphate decarboxylase gene. Host strains defective in the *URA3* gene can be used for transformation with DNA containing an intact form of the *URA3* gene, thereby enabling a ready determination of whether the desired transformation event has occurred (by return of successfully transformed cells to uracil prototrophy).

The combination of *URA3*⁻ *Pichia* strains and the *Pichia* orotidine-5'-phosphate decarboxylase marker gene provides a particularly useful selection system for use in producing recombinant strains of *Pichia* deficient in proteolytic activity. Such a selection system is referred to herein as a "bidirectional selection process". This selection system for the generation of *Pichia* strains which are deficient in proteolytic activity uses a "pop-in-pop-out" gene disruption technology in which a DNA fragment containing a defective gene is added to the genome of a host organism, with subsequent removal of

portions of the DNA fragment and endogenous sequences from the host through homologous recombination between the endogenous target gene sequence and the integrated vector sequence. Initially, transformants are selected for incorporation of the disruption vector which contains a marker gene such as *URA3* (i.e., the "pop-in" step). Next, the selected transformants must be screened to identify strains in which a recombination event between endogenous gene sequences and integrated vector sequences has occurred and has thereby excised portions of the vector, including the marker gene, and endogenous sequences of the host (i.e., the "pop-out" step). A double selection system based on the *URA3* gene and *URA3*$^-$ hosts provides for the sequential identification of the desired strains.

This type of gene disruption is typically conducted in Ura$^-$ strains, which can be identified by resistance to 5-fluoro-orotic acid (5-FOA). Disruption vectors contain a defective copy of the target gene to be disrupted and a functional *URA3* gene. Integration of the disruption vector into the genome of the Ura$^-$ host cells generates Ura$^+$ transformants containing one functional target gene and one non-functional (i.e., defective) target gene. Ura$^+$ transformants are easily identified by their ability to grow in the absence of uracil.

In order to isolate strains in which a recombination event has resulted in the elimination of the functional target gene, leaving only a defective gene, the Ura$^+$ transformants are screened for restoration of 5-FOA resistance resulting from the loss ("pop-out") of the *URA3* gene which accompanies recombination. The regeneration of the *URA3* genotype enables repetition of the "pop-in-pop-out" process for the subsequent disruption of other genes in the genome. To use this selection system for the generation of *Pichia* strains

which are deficient in proteolytic activity, a *URA3*⁻ host is transformed with a DNA construct containing a modified form of a gene encoding a protein involved in the *Pichia* proteolytic pathway, and the *URA3* gene. Site-directed integration of the transforming DNA by gene addition (i.e., "pop-in") yields one functional and one non-functional gene at the locus of the gene which directly or indirectly influences proteolytic activity, as well as an intact *URA3* gene. Strains which incorporate the *URA3* gene are identified by positive selection (using techniques well known to those of skill in the art, e.g., by growing the strains on minimal media lacking uracil and selecting those strains capable of growth on such media). The configuration of the functional, non-functional and *URA3* genes at the locus of the gene which encodes a protein which influences proteolytic activity enables recombination to occur between the functional and non-functional genes, resulting in the loss of one of these genes and the *URA3* gene (i.e., "pop-out").

Thereafter, it is possible to positively select for strains lacking a functional *URA3* gene by plating cells on medium containing a non-toxic analog of a uracil pathway intermediate, 5-fluoro-orotic acid (5-FOA), which, when metabolized by *URA3*⁺ strains, produces a compound toxic to the cells. Because *URA3*⁻ strains blocked at a specific point in the uracil pathway do not metabolize 5-FOA, they are not subjected to its toxic effects, and can thus be referred to as "5-FOA resistant". In contrast, *URA3*⁺ strains metabolize 5-FOA to produce a toxic compound which will prevent growth of the *URA3*⁺ cells. The resulting *URA3*⁻ cells that also lack the functional target gene are deficient in proteolytic activity. Because the *URA3*⁻ phenotype is restored, the resulting cells can be transformed again using the *URA3* gene as a selectable marker.

The ability to positively select strains lacking a functional *URA3* gene employing a toxic analog of a uracil pathway intermediate allows the use of this very convenient "pop-out" method for imparting multiple phenotypic changes in *Pichia* hosts.

URA3⁻ *Pichia* strains which are also deficient in proteolytic activity, relative to the proteolytic activity present in wild-type strains of the same species, are particularly useful for transformation with expression vectors which contain an intact form of the *URA3* gene, and a gene encoding a proteolytically sensitive product (either as part of the same vector, or as a second vector which is transformed into the host). Those transformants which return to uracil prototrophy (which can be readily determined by simple screening procedures) should have incorporated therein the gene encoding a proteolytically sensitive product, and thus would be directly applicable to product expression.

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

EXAMPLE I: ISOLATION OF THE *P. PASTORIS PEP4* GENE

The *P. pastoris PEP4* gene was identified in a bacteriophage lambda-based EMBL3 *P. pastoris* genomic DNA library by its ability to hybridize with a radiolabeled fragment of the homologous *Saccharomyces* cerevisiae *PEP4* gene. The *P. pastoris PEP4* gene was cloned by isolating positive plaques containing the hybridizing recombinant phage DNA.

A. Construction of a *P. pastoris* EMBL3 Genomic DNA Library

Bacteriophage λ was used as a vehicle for cloning the *P. pastoris PEP4* gene. Fragments of a partial Sau3A digest of *P. pastoris* genomic DNA were inserted into the bacteriophage λ vector EMBL3 [Frischauf, A.-M. et al.

(1983). J. Mol. Biol. 170:827], which contains elements of the bacteriophage λ genome essential for propagation of the recombinant DNA in bacterial hosts. The *P. pastoris* DNA-containing EMBL3 vectors were packaged *in vitro* into infectious virions to yield a bacteriophage λ *P. pastoris* genomic DNA library. Amplification of the library was achieved by propagation of the recombinant DNA in *Escherichia coli* host cells that had been infected with the recombinant virus.

Pichia pastoris genomic DNA (from strain NRRL Y-11430, from the Northern Regional Research Center, Peoria, IL) isolated using a glass rod swirl technique [Cregg et al. Mol. Cell. Biol. 5:3376-3385 (1985)] was digested with Sau3A at an effective concentration of 0.1 u/μg in 7, 14, 21 and 28 minute incubations conducted at 37°C. An aliquot from each incubation mixture was electrophoretically separated on a 1% agarose gel to determine the sizes of the digested DNA fragments. Digests incubated for 7 and 14 minutes appeared to consist primarily of 9-23 kb fragments. These digests were pooled and ligated to EMBL3 vector arms, prepared as described below.

EMBL3 vector arms were prepared by double digestion of the vector (obtained from EMBL3 Cloning Kit, Stratagene Cloning Systems, San Diego, CA; catalog #241211) with BamHI and EcoRI. The small BamHI/EcoRI linker that separates the arms from the stuffer fragment was removed from the digest by selective precipitation with ethanol. Ligation of the Sau3A-digested *Pichia* genomic DNA (0.5 μg) to 1 μg of EMBL3 pre-digested arms was accomplished by incubation of the 5-μl reaction mixture at 4°C for two days.

The recombinant bacteriophage λ DNA prepared by ligation of *P. pastoris* genomic DNA fragments and EMBL3 vector arms was packaged *in vitro* using commercial

packaging extracts (Stratagene EMBL3 Cloning Kit). The EMBL3-based *P. pastoris* genomic library was amplified by plating the recombinant phage along with the *E. coli* lysogenic host strain P2 392 (provided in Stratagene EMBL3 Cloning Kit) which contains prophage P2. Wild-type bacteriophage do not grow in *E. coli* strain P2 392. Recombinant EMBL3-based bacteriophage, which lack two of the wild-type genes that confer P2 sensitivity, are able to grow well in this P2-containing *E. coli* strain. The use of *E. coli* P2 392 as the host strain in the amplification ensured that only recombinant phage would be reproduced in the bacterial host.

All of the plates encompassing the EMBL3-based *P. pastoris* genomic DNA library were overlayed with SM buffer (5.8 g NaCl, 2g $MgSO_4 \cdot H_2O$, 50 ml 1M Tris·HCl, pH 7.5, and 5 ml 2% gelatin per liter). After five hours, the supernatants were collected and pooled, and the titer and genome equivalents were calculated according to the manufacturer's instructions. The library contained approximately 10 genome equivalents, and its titer was 6×10^{11} plaque-forming units/ml (pfu/ml).

B. Screening of the EMBL3 *P. pastoris* Genomic DNA Library Using the *S. cerevisiae PEP4* Gene as a Probe

In order to adequately screen the *Pichia* genome for the *PEP4* gene, 50,000 recombinant phage and the *E. coli* lysogenic host strain LE 392 (provided in Stratagene EMBL3 Cloning Kit) were plated onto four large 150-mm plates. After 6-7 hours of growth, the plates were chilled to 4°C. Each plate was marked and duplicate plaque lifts of each plate were prepared by placing nitrocellulose onto each plate. The filters were denatured, neutralized, baked and probed with the *S. cerevisiae PEP4* gene [a gel-purified, ^{32}P-labeled 4.0 kb fragment of *S. cerevisiae* DNA containing the *S. cerevisiae PEP4* gene obtained from the laboratory of Thomas Stevens, University of Oregon, Eugene, Oregon; see

Rothman et al., Proc. Natl. Acad. Sci. USA 83: 3248-3252 (1986)]. Hybridization was conducted at 37°C in a solution containing 30% formamide, 6 X SSC, 5 X Denhardt's solution, 20 mM Tris·HCl, pH 8.0, 1 mM EDTA, 0.1% SDS and 100 μg/ml salmon sperm DNA. After hybridization, the filters were washed three times at room temperature using 2 X SSC and 0.1% SDS. Following these initial washes, the filters were then washed twice at 55°C using 2 X SSC and 0.1% SDS.

Fifteen positive plaques containing DNA that hybridized to the fragment of the *S. cerevisiae PEP4* gene were identified in duplicate from autoradiograms of the filters. The area around each of the 15 positive plaques was isolated and placed in SM buffer. Six of the isolates were plated at dilutions of 10^{-5} and 10^{-7} with *E. coli* strain LE 392 onto smaller 100-mm plates. Single plaque lifts of each plate were probed with the *S. cerevisiae PEP4* gene fragment under the same hybridization and wash conditions used in the first plaque screening. In this second round of screening, 12 positive plaques were detected on the autoradiogram. Nine of these single plaques were isolated and placed in SM buffer. Each of these nine plaques was plated at dilutions of 10^{-5} and 10^{-7} with *E. coli* strain LE 392 onto small 100-mm plates. Again, single plaque lifts of each plate were probed with the *S. cerevisiae PEP4* gene fragment under the same hybridization and wash conditions used in the first two screenings. Each plate contained approximately 10-20 plaques distributed evenly across the plate. Autoradiograms of the filters revealed that every plaque on each plate hybridized to the *PEP4* probe.

Five separate plaques from different plates were isolated and placed in SM buffer. DNA from large-scale cultures of three of these isolates, designated 4721, 5111 and 5131, respectively, was prepared using the

induction method of bacteriophage isolation [Maniatis, T., Fritsch, E.F. and Sambrook, J. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA (1982)] in order to identify, characterize and subclone the *PEP4* gene contained in the recombinant phage.

C. Characterization of the Insert in Isolates of the EMBL3 *P. pastoris* Genomic DNA Library that Hybridized to the *S. cerevisiae PEP4* Gene

Recombinant phage DNA prepared from the three isolates, referred to above, from the EMBL3 *Pichia* genomic DNA library (4721, 5111 and 5131), were digested with various restriction endonucleases, separated on a 0.8% agarose gel and visualized by ethidium bromide staining. In addition, 1 µl aliquots of these digests were separated on a second agarose gel which was blotted onto nitrocellulose and probed with the radiolabeled *S. cerevisiae PEP4* gene fragment. Hybridization was conducted at 37°C in a solution containing 30% formamide, 6 X SSC, 5 X Denhardt's solution, 20 mM Tris·HCl, pH 8.0, 1 mM EDTA, 0.1% SDS and 100 µg/ml salmon sperm DNA. The filter was then washed in three 5-minute washes at room temperature with 2 X SSC and 0.1% SDS followed by two 5-minute washes at 55°C with 2 X SSC and 0.1% SDS.

Identical digests of DNA from two of the clones, 5111 and 5131, yielded the same pattern of restriction enzyme fragments, as determined by ethidium bromide staining, whereas the same digest of DNA from the third clone, 4721, yielded a different fragment pattern. Analysis of the restriction enzyme fragments of DNA from each clone by Southern blot hybridization to the *S. cerevisiae PEP4* gene fragment revealed that the two classes of clones both contained a series of hybridizing fragments of the same size indicating that the two classes of clones had a common overlapping DNA sequence that hybridized with the probe.

D. Subcloning and Characterization of the Cloned *P. pastoris PEP4* Gene

As determined by Southern blot hybridization of EcoRI-digested *P. pastoris* genomic DNA using the homologous *S. cerevisiae PEP4* gene as a probe, the *P. pastoris PEP4* gene is contained within a 10.6 kb EcoRI fragment of the *P. pastoris* genome. Southern blot hybridization of EcoRI-digested DNA of clone 4721, as described in Example 1C, revealed that it contained a 10.6 kb fragment that hybridized to the *S. cerevisiae PEP4* gene. To facilitate manipulation of the cloned *P. pastoris PEP4* gene, *P. pastoris* genomic DNA contained on an EcoRI fragment of DNA from isolate 4721 was subcloned into pUC19. Clone 4721 (25 μg) was digested with EcoRI (60 units) in a total volume of 300 μl. The digested DNA was separated on a 0.65% agarose gel, and the 10.6 kb EcoRI fragment was isolated with DE81 paper. The purified fragment was washed from the paper with 400 μl of 1 M NaCl and extracted with phenol/chloroform. The DNA was then precipitated with ethanol and resuspended in water to a total volume of 10 μl. Approximately 50 ng of the 10.6 kb fragment were ligated with an equal amount of pUC19 which had been cut with EcoRI and dephosphorylated. The ligation mixture was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and screened by analysis of restriction enzyme digests of colony DNA for the presence of the diagnostic 10.6 kb EcoRI fragment. A large-scale plasmid preparation was made from a colony containing the correct plasmid, which was named pEP202. Plasmid pEP202 contains the complete *P. pastoris PEP4* gene (see Figure 2).

To facilitate sequence analysis of the cloned *P. pastoris PEP4* gene, a portion of the *P. pastoris PEP4* gene was subcloned into pUC19. Plasmid pEP202 was digested with BamHI and EcoRI. The reaction mixture was separated on a 0.7% agarose gel, and the 0.45 kb BamHI

fragment of DNA (see Fig. 2) was isolated using DE81 paper. The purified fragment was ligated to pUC19 (~20 ng) that had been linearized by digestion with BamHI and dephosphorylated. The ligation mixture was used to transform *E. coli* strain MC1061. Transformants were selected for ampicillin resistance and screened by analysis of restriction enzyme digests of colony DNA for the presence of a single BamHI fragment. A single colony arising from this transformation was found to contain the appropriate DNA construct, and was named pEP205 (see Figure 3).

Sequence analysis of plasmid pEP202 identified a DNA sequence with ~70% homology to the *PEP4* gene of *S. cerevisiae*. The amino acid sequence encoded by this DNA sequence of pEP202 is 69% homologous to that encoded by the *S. cerevisiae PEP4* gene.

EXAMPLE II: DEVELOPMENT OF A *PEP4*-DEFICIENT (*PEP4*$^-$) STRAIN OF *P. PASTORIS*

A. Construction of the *P. pastoris PEP4* Gene Disruption Vector pDR401

Vector pDR401 was constructed for use in developing a *PEP4*-deficient (*PEP4*$^-$) strain of *P. pastoris*. This vector contains a defective *P. pastoris PEP4* gene, which, when used to transform *PEP4* strains of *P. pastoris*, integrates into the host genome by replacement of the wild-type *PEP4* gene.

pDR401 was constructed in a two-step procedure as follows. In the first step, the base vector in the construction of pDR401, base vector pEP301, was constructed from pEP202. Vector pEP301 constains pUC19 sequences and the cloned *P. pastoris PEP4* gene from pEP202. Plasmid pEP202 (15 μg) was digested with SacI. A 5.5 kb SacI fragment (the fragment extending from the SacI linker clockwise to the SacI site at ~5:00, and containing all of the pUC19 sequence and the entire *PEP4* gene; see Figure 2) was isolated from a 0.7% agarose gel

using DE81 paper. The fragment was eluted from the paper with 400 µl of 1 M NaCl, extracted with 400 µl of phenol/chloroform and precipitated with ethanol. This DNA was then ligated with itself in a volume of 100 µl containing 1 µl of ligase and 1 µl (~10 ng) of DNA. The ligation mixture was incubated at room temperature for 1 hr and then used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and screened by analysis of restriction enzyme digests of colony DNA for the presence of a single 5.5 kb BglII fragment. Plasmid DNA was prepared from a transformed colony of MC1061 that contained the correct plasmid, which was named pEP301 (Figure 4).

In the second step of the construction of pDR401, the *P. pastoris HIS4* gene was inserted into the *PEP4*-containing plasmid pEP301 to yield the final vector. The *P. pastoris HIS4* gene was isolated on a 2.6 kb BglII fragment derived from pYJ8ΔCla [Cregg, J. et al. Mol. Cell. Biol. 5:3376-3385 (1985)]. Plasmid pYJ8ΔCla (15 µg) was digested with BglII and the digested DNA was separated on a 0.7% agarose gel. The *HIS4* gene-containing 2.6 kb fragment was isolated with DE81 paper, eluted with 400 µl of 1 M NaCl, extracted with 400 µl of phenol/chloroform, precipitated with ethanol and resuspended in 10 µl of water. Prior to inserting this 2.6 kb BglII fragment into the unique BglII site of pEP301, approximately 20 µg of pEP301 were digested with BglII, dephosphorylated and extracted with phenol/chloroform. The 2.6 kb *HIS4*-containing fragment was then inserted into pEP301 by ligation of approximately 50 ng of the fragment to approximately 50 ng of the BglII-digested pEP301 in a total volume of 10 µl containing 1 µl of buffer, 1 µl of ligase and water. Ligation was conducted at room temperature for 3 hrs and the ligation mix was used to transform MC1061 cells. Plasmid DNA

prepared from an ampicillin-resistant colony was digested with BglII, SalI, BglII/SalI, PvuI, NcoI and KpnI to confirm the construction of pDR401. The restriction fragment pattern was consistent with that expected for the correct plasmid pDR401 (see Figure 5). Plasmid pDR401 is pUC19 with the *P. pastoris HIS4* gene inserted at the unique BglII site within the *PEP4* structural gene, thus disrupting it.

B. Transformation of *HIS4 P. pastoris* Strain GS115 with a Fragment of pDR401

In order to create a *PEP4* strain of *P. pastoris*, the *HIS4 PEP4 P. pastoris* strain GS115 (ATCC 20864) was transformed with 20 μg of the 5.3 kb EcoRI/SacI fragment of pDR401 according to the spheroplast method (see US patent 4,879,231). This fragment of pDR401 consists of the *HIS4* gene-containing defective *PEP4* gene. Transformant strains resulting from this type of integration are prototrophic and can be distinguished from untransformed cells on this basis. The frequency of transformation was approximately 10^3 μg^{-1} DNA.

C. Characterization of Transformants

1. Analysis of transformant carboxypeptidase Y activities

His^+ transformants were subsequently analyzed for carboxypeptidase Y activity using a colony overlay colorimetric screening procedure [see Jones, E. in Genetics 85: 23-33 (1977)]. In this assay, the His^+ transformant cells were released from the transformation agar plates and grown on YEPD (yeast extract, 1% peptone, 2% dextrose and 2% agar) plates at a density of ~300 colonies per plate. The plates were overlayed with 0.6% agarose containing 40% dimethylformamide (DMF) to permeabilize the cells, and 1.2 mg/ml of the substrate APNE (N-acetyl DL phenylalanine β-naphthyl ester). Because the cells were permeabilized, some of the vacuolar content of the cell was accessible to the

reagent APNE. After the agarose overlay had solidified, the plates were soaked in a solution of 5 mg/ml Fast garnet salt. APNE is cleaved by the esterolytic activity of carboxypeptidase Y. The products of this reaction bind the fast garnet salt to produce a red color in the colony. Colonies lacking carboxypeptidase Y activity do not bind the salt and therefore stain less intensely than do colonies that possess this activity. *PEP4*$^+$ colonies developed a red/pink center during the first 10-15 minutes after exposure to the garnet salt. In contrast, colonies defective at the *PEP4* locus were slow to develop this color and were distinguished as pink relative to the red *PEP4*$^+$ colonies. Colonies that appeared to have low carboxypeptidase Y activities based on the results of this assay (i.e., colonies that failed to develop a strong red color indicative of *PEP4*$^+$ colonies) were isolated, transferred to a master plate, subcultured along with control colonies and re-screened using the overlay assay. Twenty colonies which again failed to develop a strong red color were selected for analysis by Southern blot hybridization to determine if the *PEP4* locus of these transformants had been disrupted by integration of the fragment of vector pDR401.

2. **Southern blot hybridization analysis**

Genomic DNA was extracted from 20 transformant strains that exhibited low carboxypeptidase activity, designated p1-p20, and digested with SacI and EcoRI. This procedure should liberate the *HIS4*-containing defective *PEP4* gene as the 5.3 kb EcoRI/SacI fragment that was used to transform the strains. Two Southern blot filters were prepared from these digested DNAs; one blot was probed with a radiolabeled 1.4 kb XbaI/EcoRV fragment from pEP301 (see Fig. 4), which contained a portion of the cloned *P. pastoris PEP4* gene and the other blot was probed with a radiolabeled 2.6 kb BglII fragment of

pDR401 containing the *HIS4* gene. Control DNA from the transformation host strain GS115, which had been digested with SacI and EcoRI, was included in this analysis for comparative purposes.

Digestion of genomic DNA from GS115 with SacI and EcoRI yielded a 2.9 kb fragment that hybridized to the portion of the *PEP4* gene contained in the radiolabeled XbaI/EcoRV fragment of pEP301. In contrast, this probe hybridized to fragments of a different size in SacI/EcoRI-digested DNA from 19 of the 20 transformants analyzed. Only DNA from strain p17 yielded a hybridization pattern identical to that of DNA from the parental strain. The remaining 19 strains lacked a 2.9 kb hybridizing fragment characteristic of an undisrupted *PEP4* locus and contained an approximately 5.3 kb fragment and/or larger fragments that hybridized to the *PEP4* gene probe. The 5.3 kb fragment was the same size as the transforming DNA released from vector pDR401 upon digestion with SacI and EcoRI.

The results of Southern blot hybridization of DNA from strains p1-p16 and p18-p20 revealed that these strains contained a defective *PEP4* gene with an intact *HIS4* gene therein, and that the *PEP4* locus of the strains had been disrupted. Strain p13 was grown in a one-liter fermentation, as described in Example III, in order to analyze the proteolytic activity of the broth of a larger culture of a *PEP4* strain of *P. pastoris*.

3. Analysis of the transformant proteinase A activities

a. Protocol

The proteinase A activities of eight transformant strains were evaluated using an enzyme assay based on the method of Jones et al. [Genetics 102:655 (1982)]. Several control strains were also evaluated in this assay: *PEP4* and *PEP4* strains of *S. cerevisiae* (strains DBY747 and 20B12, respectively, from the Yeast Genetic

Stock Center, University of California, Berkeley, CA) and a *PEP4* wild-type strain of *P. pastoris* (strain NRRL Y-11430 from the Northern Regional Research Center, Peoria, IL).

Proteinase A is a vacuolar enzyme responsible for the aspartyl protease activity encoded by the *PEP4* gene in *S. cerevisiae*. The procedure used to evaluate the proteinase activities of transformant cell extracts is based on the measurement of proteinase A-mediated release of amino acids from acid-denatured hemoglobin. Transformant cell extracts were incubated with acid-denatured hemoglobin, and the proteinase A activity present in the extract was determined by estimating the difference in the amount of amino acid released at time zero and after 90 minutes of incubation.

Cultures of the *S. cerevisiae* control strains DBY747 (*PEP4*) and 20B12 (*PEP4*), the *PEP4 P. pastoris* strain NRRL Y-11430 and the experimental *PEP4* strains of *P. pastoris* were grown to stationary phase in YEPD medium. Cultured cells (20 OD_{600} units) were washed in 10 mM sodium azide and then lysed in 400 µl of 100 mM Tris, pH 7.5, by vortexing the cells with acid-washed glass beads for one minute. The lysed cells were centrifuged in Eppendorf tubes for 10 minutes to remove cell debris. The supernatant obtained after centrifugation (crude extract) was then examined for proteinase A activity as follows. Acid-denatured 1% hemoglobin (400 µl) was added to 50 µl of crude extract and incubated for 90 minutes at 37°C. Reactions were stopped by the addition of 0.2 ml of 1 N perchloric acid. Insoluble material was removed by centrifugation, and 200 µl of 0.31 M NaCl was added to 200 µl of supernatant. A 40 µl aliquot of this solution was then assayed using the Pierce BCA protein assay kit (see, for example, US Patent No. 4,839,295) for free amino acids. The amount of free amino acids present in

the sample that had been incubated for 90 minutes was compared to the amount present in a blank which consisted of a sample of a reaction mixture that was stopped at zero minutes. The relative difference in free amino acids between these two samples is a measure of proteinase A activity.

b. Results

The results of proteinase A assays of control and transformant strains (see Table I; ΔOD is a measure of the concentration of free amino acids in the sample) indicate that the proteinase A activity of the *PEP4* strain of *S. cerevisiae* represents only 10% of that of the *PEP4* strain of *S. cerevisiae*. Similarly, the proteinase A activities of the *PEP4* transformant strains (strains p1, p2, p5, p8, p13, p16 and p20) also are only approximately one-tenth of that of the *PEP4* strain of *S. cerevisiae*. The *PEP4* wild-type strain of *P. pastoris* displayed approximately half of the proteinase A activity of the *PEP4* strain of *S. cerevisiae*.

TABLE I
PROTEINASE A ASSAY RESULTS

Strain	Phenotype	ΔOD/μg protein
DBY747 (*S. cerevisiae*)	*PEP4*$^+$	28.1
20B12 (*S. cerevisiae*)	*PEP4*$^-$	2.7
P. pastoris control (NRRL Y-11430)	*PEP4*$^+$	13.1
p13	*PEP4*$^-$	3.3
p20	*PEP4*$^-$	4.2
p17	*PEP4*$^+$ (?)	7.5
p16	*PEP4*$^-$	0
p16	*PEP4*$^-$	0
p13	*PEP4*$^-$	3.3
p8	*PEP4*$^-$	3.3
p5	*PEP4*$^-$	5.0
p2	*PEP4*$^-$	6.6
p1	*PEP4*$^-$	6.0

The data obtained in proteinase A assays of *PEP4* *P. pastoris* strains generated by transformation of a *PEP4* strain with a defective *PEP4* gene are consistent with the results of Southern blot analyses of DNA from these transformants which indicate that the *PEP4* locus of the transformants was disrupted upon transformation.

EXAMPLE III: FERMENTATION OF A *PEP4* STRAIN OF *P. PASTORIS*

A. Procedure

A *PEP4* strain of *P. pastoris*, p13, generated by transformation of strain GS115 with a defective *PEP4* gene-containing SacI/EcoRI fragment of vector pDR401, was grown in a one-liter fermentation according to a three-phase protocol consisting of a glycerol batch growth phase, a limited glycerol fed-batch phase and a methanol fed-batch phase as follows.

A two-liter fermentor was autoclaved with 1000 ml of minimal salts medium (21 ml 85% phosphoric acid, 0.9 g calcium sulfate·$2H_2O$, 14.3 g potassium sulfate, 11.7 g magnesium sulfate and 3.2 g potassium hydroxide) and 2% glycerol. After sterilization, 4 ml PTM_1 trace salts solution (6 g/l cupric sulfate·$5H_2O$, 0.8 g/l sodium iodide, 3 g/l manganese sulfate·H_2O, 0.2 g/l sodium molybdate·$2H_2O$, 0.02 g/l boric acid, 0.5 g/l cobalt chloride, 20 g/l zinc chloride, 65 g/l ferrous sulfate·H_2O, 0.2 g/l biotin and 5 ml sulfuric acid) were added to the fermentor and the pH was adjusted to 5 with concentrated NH_4OH. The pH of the medium was maintained at 5 by addition of 50% NH_4OH containing 0.1% Struktol J673 antifoam. Inocula were prepared from buffered yeast nitrogen base (YNB) glycerol plates (phosphate-buffered YNB, 2% glycerol, 2% agar) and grown overnight at 30°C in phosphate-buffered YNB (11.5 g/L KH_2PO_4, 2.66 g/L K_2HPO_4, 0.67% yeast nitrogen base, pH 5) containing 2% glycerol. The fermentor was inoculated with 10-50 ml of the cultured cells which had grown to an OD_{600} of 1-8, and the

batch growth regimen was continued for approximately one day until glycerol was exhausted. At the point of glycerol exhaustion, as indicated by increased dissolved oxygen, a glycerol feed (50% glycerol plus 12 ml/L of PTM_1) was initiated at 10 ml/h and continued until 40 ml of glycerol feed had been added. After termination of the glycerol feed, a methanol feed (100% methanol plus 12 ml/L PTM_1) was started at an initial rate of approximately 2 ml/h. After 3 hours, the methanol feed rate was increased to 6 ml/h. The methanol feed rate was maintained at 6 ml/h for 12-18 hours and was then increased to 10 ml/h and maintained at 10 ml/h for the duration of the fermentation. The vessel was harvested after 400 ml of methanol had been added to the fermentor.

B. **Sample Preparation**

Samples (15 ml aliquots) of the fermentor culture were removed from the fermentor at various time intervals throughout the course of the fermentation. Aliquots of each sample were centrifuged at 6500 x g for 5 minutes to separate broth and cells. The levels of the NH_4OH, antifoam, glycerol, and methanol reservoirs were recorded at these time points. Methanol and ethanol concentrations in the supernatant were determined by gas chromatography using a PorapakQ column (Alltech).

In addition, the wet weight of the culture was determined as an indicator of cell growth in the fermentor. For this purpose, a one ml aliquot of the fermentor culture was centrifuged for four minutes in a microfuge, the supernatant was decanted, and the wet pellet was weighed.

C. **Results**

Growth of the *PEP4* strain of *P. pastoris* p13 in a one-liter fermentation was monitored by determining the wet cell weight of the fermentor culture (in g/l) at various times during the fermentation. A time course of

the growth of strain p13 during the methanol fed-batch phase of the fermentation, when compared with the time course of the growth of the *HIS4 PEP4* strain G+PAO804H2 (generated by transformation of the *HIS4 PEP4 P. pastoris* strain GS115 with an expression vector containing the wild-type *HIS4* gene) during a similar one liter fermentation, demonstrates that the growth capabilities of the *PEP4* strain of *P. pastoris* are comparable to those of a *PEP4* strain.

EXAMPLE IV: ANALYSIS OF THE PROTEOLYTIC ACTIVITY OF THE BROTH OF A *PEP4* STRAIN OF *P. PASTORIS* GROWN IN A ONE-LITER FERMENTATION

To determine if disruption of the *P. pastoris PEP4* gene was associated with a change in the proteolytic activity of the broth of *P. pastoris*, the proteolytic activities of the broths from one-liter fermentations of a *PEP4* strain, strain p13, and a *PEP4* strain were compared. In this study, two different peptides, epidermal growth factor (EGF; a recombinantly synthesized molecule consisting of the first 52 amino acids of the authentic 53 amino acid EGF molecule, as described in US patent application Serial No. 323,964) and growth hormone releasing factor (GRF; recombinantly synthesized as described in EP 206783) were separately incubated at room temperature in cell-free broth from the one-liter fermentation of the *PEP4 P. pastoris* strain p13, and in the cell-free broth from a similar one-liter fermentation of the *HIS4 PEP4 P. pastoris* strain G+PAO804H2. After incubation for a specified period, aliquots of each incubation mixture were examined by reverse phase high performance liquid chromatography (HPLC), described below, to determine the amount of intact peptide remaining in each sample and thereby determine the extent of proteolytic degradation of the peptide.

A. **Reverse-Phase High-Performance Liquid Chromatography (HPLC)**

The reverse-phase HPLC system used in the analysis of EGF and GRF peptides in buffer and broth from fermentations of *P. pastoris* strains included a Waters 600 (Bedford, MA) solvent delivery system, Waters Model 481 Lambda Max variable wavelength detector, Wisp 710B autoinjector and a Shimadzu Chrom-Pac integrator (Cole Scientific, Moorepark, CA). Samples of broth from the fermentations of the *PEP4 P. pastoris* strain p13 and the *HIS4 PEP4 P. pastoris* strain G+PAO804H2 were diluted 1:10 in 0.1 M sodium phosphate, pH 5.0. Fifteen microliters of concentrated GRF stock was added to 285 μl of diluted broth and incubated for four hours. A similar dilution of GRF stock in the phosphate buffer was also incubated for four hours as a control. Sixty microliters of EGF stock were added to 240 μl of diluted broth or buffer and incubated for eight hours. Samples of each incubation mixture were separately injected into a Waters μ Bondapak C18 reverse phase column. The peptides were eluted from the column in a 20-minute linear gradient of 20-60% mobile phase B (95% acetonitrile, 5% water, 0.1% trifluoroacetic acid). Mobile phase A (0.1% trifluoroacetic acid) was used to dilute mobile phase B in preparing the elution gradient.

B. **Results**

The amount of intact peptide (of the EGF or GRF molecules that were incubated in the fermentation broth of the *PEP4 P. pastoris* strain p13 and the broth of the *PEP4 P. pastoris* strain G+PAO804H2) was evaluated by comparing chromatograms obtained in HPLC analyses of intact EGF or GRF contained in 0.1 M sodium phosphate buffer, pH 5.0, and of EGF or GRF contained in broth. Chromatograms from HPLC analyses of the standard intact peptides consist of a major peak reflecting the amount of the standard peptide present in the sample and the

retention time characteristic of the peptide. In contrast, proteolytic fragments of either peptide are retained on the HPLC column for varying lengths of time that differ from the retention time associated with the intact peptide. Therefore, chromatograms from HPLC analysis of proteolytic fragments of either peptide (EGF or GRF) differ from chromatograms generated in HPLC analyses of intact peptides in terms of the number and sizes of the peaks and the retention times associated with the fragmented species. Based on these differences, it was possible to estimate the amount of intact EGF or GRF peptide in the broth incubation samples.

Based on HPLC analyses of GRF and EGF samples incubated in *PEP4 P. pastoris* control broth, it has been determined that less than 10% of each of the two peptides remains intact after incubation in broth from the fermentation of the *PEP4* strain G+PAO804H2. In contrast, the level of proteolytic degradation of these peptides in the broth of the *PEP4 P. pastoris* strain is significantly less than that in the broth of the *PEP4* strain (GRF remained >60% intact, even after 4 hr incubation; EGF remained >90% intact, even after 8 hr incubation). These data demonstrate that disruption of the *PEP4* gene of *P. pastoris* results in a substantial reduction of the proteolytic activity in the broth of the strain.

EXAMPLE V: **ISOLATION OF THE *P. PASTORIS URA3* GENE**

The *P. pastoris URA3* gene was identified in a plasmid (YEp13)-based *Pichia* genomic library by its ability to complement the pyrF mutation (corresponding to a defect in the orotidine monophosphate decarboxylase activity) in *E. coli* strain CSH-28. The *P. pastoris URA3* gene was cloned by isolating colonies of *E. coli* strain CSH-28 that had been transformed with library DNA and were capable of growth on media lacking uracil.

A. *P. pastoris* YEp13 Genomic DNA Library

Plasmid YEp13 [Broach et al., Gene 8: 121-133 (1979)] is a convenient shuttle vector that contains an origin of replication for both *S. cerevisiae* (2μ replicon) and *E. coli* (pBR ori). In addition, YEp13 contains the Amp^R (ampicillin resistance) gene for use as a selectable marker for transformation of *E. coli* and the *LEU2* gene (a leucine biosynthetic pathway gene) for use as a selectable marker in *S. cerevisiae*. A *P. pastoris* (strain NRRL Y-11430) genomic DNA library has been prepared using plasmid YEp13, as described by Cregg et al. [Mol. Cell. Biol. 5: 3376-3385 (1985)].

B. Screening of the *P. pastoris* YEp13 Genomic DNA Library for the *URA3* Gene

The pyrF *E. coli* strain CSH-28 [see Miller, J. H., in Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1972)] is defective for orotidine-5'-phosphate decarboxylase activity and requires uracil when grown on defined medium. It has been demonstrated that the S. cervisiae *URA3* gene can complement the pyrF mutation in *E. coli* [Rose, M., Grisafi, P. and Botstein, D. Gene 29:113-124 (1984)]. Therefore, *E. coli* strain CSH-28 was transformed with DNA from the *P. pastoris* YEp13 genomic DNA library in order to screen the library for the *P. pastoris URA3* gene capable of complementing the pyrF mutation of the strain.

Transformed CSH-28 cells were plated onto a semi-defined medium which did not contain uracil. Untransformed cells would not grow on this medium. CSH-28 transformants (transformed with *P. pastoris* genomic library DNA) capable of growing on plates lacking uracil arose at a frequency of ~10/μg of transforming DNA. Plasmid DNA was isolated from ten of the transformants that did not require uracil for growth. These plasmids were used to transform *E. coli* strain

CSH-28, and ten of ten plasmids complemented the uracil auxotrophy of this strain at high frequency. One of the selected transformants generated by transformation of CSH-28 with *P. pastoris* genomic library DNA harbored a 9.0 kb insert that contained a 6.6 kb SphI fragment. The 6.6 kb SphI fragment was subcloned into the SphI site of pUC19 for further analysis.

Plasmid DNA from this transformant was digested with SphI and subjected to electrophoresis on a 0.6% agarose gel. The 6.6 kb fragment was isolated using DE81 paper and was eluted from the paper with 400 µl of 1 M NaCl. DNA was extracted with 400 µl of phenol/chloroform and precipitated with ethanol. The 6.6 kb fragment was then ligated with 10 ng of alkaline phosphatase-treated, SphI-digested pUC19. The ligation mixture was used to transform *E. coli* MC1061 cells. Ampicillin-resistant transformants were screened by analysis of restriction enzyme-digested colony DNA for the presence of a 6.6 kb SphI fragment. The correct plasmid was called pPU201. Plasmid pPU201 was used to transform CSH-28 and was able to complement the uracil auxotrophy of this strain.

C. Characterization of the Insert in Plasmid pPU201

A map of the restriction enzyme recognition sites of the 6.6 kb insert of *P. pastoris* DNA in plasmid pPU201 (Figure 6) was prepared by digesting pPU201 with a variety of enzymes and analyzing the resulting fragments using a DNA length computer program (MapSort; University of Wisconsin Genetics, Madison, WI) to determine the approximate sizes of the fragments. In order to delineate the *URA3* gene contained in the 6.6 kb insert of pPU201, a 5 ng aliquot of each restriction enzyme digest of pPU201 was separated by electrophoresis on a 1% agarose gel, transferred to nitrocellulose, and probed with a radiolabeled 1.3 kb BglII fragment of the *C. tropicalis URA3*A gene (see PCT Publication No. WO

90/09449). The filters were hybridized to the probe at 27°C using a solution containing 25% formamide, 6x SSC, 5x Denhardt's solution, 20 mM Tris·HCl, pH 8.0, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS) and 100 μg/ml salmon sperm DNA. After hybridization, the filters were washed three times at room temperature using 1x SSC and 1% SDS for 5-10 minutes per wash, and then washed twice with 0.5x SSC and 0.5% SDS at 45°C for 10 minutes per wash. These low stringency conditions permitted hybridization between divergent *URA3* gene sequences. Additional samples of each digest of pPU201 were separated on an identical 1% agarose gel and stained with ethidium bromide for comparison of hybridizing and non-hybridizing fragments. Comparison of the hybridizing fragments and the restriction map of pPU201 made it possible to localize the *URA3* gene in pPU201 to the approximately 1.3 kb NcoI-SalI fragment as shown in Figure 6. With this knowledge, it was then possible to construct subclones suitable for sequencing and further characterization of the *P. pastoris URA3* gene.

Plasmid pPU202 (Figure 7) was constructed by digesting pPU201 with EcoRV and PstI, isolating the approximately 4.0 kb fragment containing the *URA3* gene, and ligating it into pUC19 at the SmaI and PstI sites. Plasmids pPU203, pPU205 and pPU206 (Figures 8-10) were constructed by digesting pPU202 with SacI, KpnI and EcoRI, respectively, and then religating in a large volume (200 μl). Because there is a recognition site for each of these enzymes in the cloned *P. pastoris* genomic insert DNA fragment as well as the pUC19 polylinker of pPU202, this strategy allowed for the convenient removal of DNA between these sites in pPU202. The resulting plasmids were then used to transform *E. coli* strain CSH-28 to determine whether or not each deletion construct could complement the pyrF mutation. The results

indicated that pPU203 and pPU205, but not pPU206, contained a functional *URA3* gene allowing growth of the pyrF strain on defined medium lacking uracil. These findings are consistent with the mapped position of the *P. pastoris URA3* gene in pPU201.

The subclones of the *P. pastoris* genomic DNA fragment carrying the putative *URA3* gene were sequenced using the Sanger dideoxy method [see Sanger et al., Proc. Natl. Acad. Sci. USA 74: 5463-5467 (1977)]. The sequence for the structural gene and approximately 100 bp of flanking sequence was determined in both directions and is presented in Sequence ID No. 3. The amino acid sequence deduced from the cloned *P. pastoris URA3* gene (see Sequence ID No. 4) has 73% homology with the amino acid sequence deduced from the *S. cerevisiae URA3* gene, 71% homology with the amino acid sequence deduced from the *URA3*A and *URA3*B genes of *C. tropicalis* and 72% homology with the amino acid sequence deduced from the *URA3* gene of *Kleuveromyces lactis*.

EXAMPLE VI: DEVELOPMENT OF IGF-1-EXPRESSING *PEP4*-DEFICIENT (*PEP4*$^-$) STRAINS OF PICHIA

A. Generation of IGF-1-Expressing *PEP4*$^-$ Strains by Gene Addition

1. Construction of the *P. pastoris PEP4* gene disruption vector pDR421

Plasmid pDR421 was constructed for use in the development of *PEP4*-deficient (*PEP4*$^-$) strains of *Pichia pastoris* by disruption of a host *PEP4* gene through addition of an incomplete *PEP4* gene to the endogenous *PEP4* locus. This vector contains an internal portion of the *PEP4* gene, which, when used to transform *PEP4* strains of *P. pastoris*, integrates into the host genome at the *PEP4* locus to generate two incomplete and nonfunctional copies of the *PEP4* gene.

In order to generate the disruption vector pDR421, the *URA3* gene of *Pichia* was cloned into vector pEP205

(consisting of pUC19 sequences and the portion of the *PEP4* gene contained in the ~450 bp BamHI fragment derived from pEP202). This was achieved by subcloning the *URA3* gene from pPU205 (see Figure 9) as a 2 kb SpeI-SphI DNA fragment into the XbaI-SphI sites of pEP205 (see Fig. 3).

Plasmid pPU205 was digested with SpeI and SphI and the reaction mixture was separated on a 0.8% agarose gel. The 2 kb DNA fragment containing the *URA3* gene was isolated from the gel using DE81 paper, eluted and purified. Plasmid pEP205 was digested with XbaI and SphI. The 2 kb *URA3* gene-containing SpeI-SphI fragment isolated from pPU205 was ligated to XbaI/SphI-digested pEP205 and the mixture was used to transform *E. coli* strain MC1061 to ampicillin resistance. Ampicillin-resistant colonies were screened by analysis of BamHI/SphI restriction enzyme-digested colony DNA for the presence of 2.7 kb, 0.4 kb and 1.9 kb diagnostic fragments. A transformant was found to harbor a plasmid with the correct DNA construct called pDR421 (Figure 11).

2. **Transformation of an IGF-1-expressing *URA3* *P. pastoris* strain (IGF-U) with pDR421**

The *URA3* IGF-1-expressing strain of *P. pastoris*, IGF-U, was transformed with pDR421 to generate *PEP4*$^-$, IGF-1-expressing strains of *P. pastoris*.

a. **Generation of IGF-U**

5-Fluoro-orotic acid (5-FOA) is an analog of a uracil biosynthetic pathway intermediate that, when metabolized by Ura$^+$ strains, yields a toxic compound. Because the uracil biosynthetic pathway of Ura$^-$ strains is blocked at certain steps, these strains do not metabolize 5-FOA (to produce a compound toxic to the cells) and are therefore 5-FOA resistant. In contrast, Ura$^+$ strains metabolize 5-FOA and cannot survive on 5-FOA-containing medium. Therefore, plating cells on 5-FOA-containing medium can be used as a method to generate Ura$^-$ strains by

spontaneous mutation [see, for example, Boeke et al., Mol. Gen. Genet. 197: 345-346 (1984)].

A *URA3*⁻ derivative of the IGF-1-producing strain G+IMB206S1 [for a description of this strain, see commonly assigned U.S. Patent Application Serial No. 07/578,728, filed September 4, 1990,which is hereby incorporated by reference herein in its entirety] was generated by direct plating of ~5 x 10^7 cells this strain into 5-FOA-containing medium supplemented with uracil (0.67% yeast nitrogen base, 2% agar, 2% glucose, 750 mg/l of 5-FOA and 48 mg/l of uracil). After one week of incubation at 30°C, a colony, designated IGF-U, growing on the plate was isolated. This colony, which required uracil in order to grow, was unable to complement a *URA3* strain of *Pichia pastoris*.

b. Transformation of IGF-U

Approximately 20 μg of pDR421 was digested with BglII was used to transform IGF-U using the standard spheroplast transformation procedure. Transformants were selected by their ability to grow in the absence of uracil over a 6 day period.

3. Characterization of transformants

a. Analysis of transformant carboxypeptidase Y activities

Ura⁺ transformants were subsequently analyzed for carboxypeptidase Y activity using a colony overlay colorimetric screening procedure, as described in Example II. Colonies of Ura⁺ transformants that appeared to have low carboxypeptidase Y activities based on the results of this assay (i.e., colonies that failed to develop a strong red color indicative of *PEP4*⁺ colonies) were isolated, transferred to a master place, subcultured along with control colonies and rescreened using the overlay assay. One colony which again failed to develop a strong red color was called M+IMB206S1.

b. Analysis of intact IGF-1 expression levels of an IGF-1-expressing *PEP4* strain of *P. pastoris* grown in one- and ten-liter fermentations

i. Fermentation of an IGF-1-expressing *PEP4* strain of *P. pastoris*

An IGF-1-expressing *PEP4* strain of *P. pastoris*, M+IMB206S1, generated as described in Example VI.A.2.b., was grown in one- and ten-liter fermentations according to a three-phase protocol consisting of a glycerol batch growth phase, a limited glycerol fed-batch phase and a methanol fed-batch phase. In order to compare the intact IGF-1 expression levels of *PEP4* and *PEP4* IGF-1-expressing strains of *P. pastoris*, two *PEP4* strains of *P. pastoris*, G+IMB204S14 and G+IMB206S1, containing four and six copies of an IGF-1 gene expression cassette, respectively (see, U.S. application Serial No. 07/578,728), were also grown in comparable fermentations as follows.

One-liter fermentation protocol

A two-liter fermentor (Biolafitte, Princeton, NJ) was autoclaved with 900 ml of minimal salts medium (21 ml 85% phosphoric acid, 0.9 g calcium sulfate·$2H_2O$, 14.3 g potassium sulfate, 11.7 g magnesium sulfate, and 3.2 g potassium hydroxide) and 30 g of glycerol. After sterilization, 4 ml PTM_1 trace salts solution (6 g/l cupric sulfate·$5H_2O$, 0.08 g/l sodium iodide, 3 g/l manganese sulfate·H_2O, 0.2 g/l sodium molybdate·$2H_2O$, 0.02 g/l boric acid, 0.5 g/l cobalt chloride, 20 g/l zinc chloride, 65 g/l ferrous sulfate·H_2O, 0.2 g/l biotin and 5 ml sulfuric acid) were added to the fermentor and the pH was adjusted to 5 with concentrated NH_4OH. The pH was controlled by addition of 50% NH_4OH containing 0.1% Struktol J673 antifoam (added to control foaming). The temperature was maintained at 30°C, and dissolved oxygen was maintained above 20% of saturation by increasing agitation, aeration, or the supplementation of the air feed with oxygen.

Inocula were prepared from cells grown overnight at 30°C in buffered YNB containing 2% glycerol. The fermentor was inoculated with 40-70 ml of the cultured cells which had grown to an OD_{600} of 2-8, and the batch growth regimen was continued for 18-24 hours until glycerol was exhausted. At the point of glycerol exhaustion, indicated by an increase in dissolved oxygen concentration, a glycerol feed (50% w/v glycerol plus 12 ml/L PTM_1) was initiated at 10 ml/hr. In pH 5.0 fermentations, the pH of the culture was maintained at 5 throughout the fermentation. In low pH fermentations (i.e., pH 2.8 or pH 3.5), the set point of the pH controller was adjusted to the desired pH after initiation of the glycerol feed. After four hours, the pH of the culture decreased to the set point value as a result of cellular metabolism. This lower pH was then maintained throughout the remainder of the fermentation. The glycerol feed was then terminated and a methanol feed (100% methanol plus 12 ml/L PTM_1) was initiated at a rate of 2 ml/hr. After three hours of methanol feeding, the feed rate was increased to 6 ml/hr and maintained at this rate for the remainder of the fermentation. The vessel was harvested 72 hours after initiation of the methanol feed.

The fermentation was monitored in terms of NH_4OH, antifoam, glycerol, methanol, ethanol, and wet cell weight levels as described in Example III. Broth and cell samples were collected throughout the fermentation as also described in Example III.

Ten-liter fermentation protocol

A 15-liter fermentor containing 3.5 liters of 10X basal salts (42 ml 85% phosphoric acid/l, 1.8 g calcium sulfate·$2H_2O$/l, 28.6 g potassium sulfate/l, 23.4 g magnesium sulfate/l, 6.5 g potassium hydroxide/l) and 220 g glycerol in a total volume of 5.5 liters was

sterilized. After the fermentor had cooled, 24 ml PTM_1 trace salts were added and the pH was adjusted to 5.0 with the addition of 28% ammonium hydroxide. The pH was controlled by the addition of the same solution. Foaming was controlled with the addition of a 5% solution of Struktol J673. Temperature was maintained at 30°C, and dissolved oxygen was maintained above 20% of saturation by increasing agitation, aeration, reactor pressure or by supplementation of the air feed with oxygen. Inocula were prepared from *P. pastoris* cells grown overnight in buffered yeast nitrogen base (YNB; 11.5 g/L KH_2PO4, 2.66 g/L K_2HPO_4, 6.7 g/L yeast nitrogen base, pH 6) containing 2% glycerol. The fermentor was inoculated with 500-700 ml of the cultured cells which had grown to an OD_{600} of 2-8, and the batch growth regime was continued for 18-24 hours. At the point of glycerol exhaustion, indicated by an increase in dissolved oxygen concentration, a glycerol feed (50% w/v glycerol plus 12 ml/L PTM_1) was initiated at 100 ml/hour and continued for 4 hours. The glycerol feed was then terminated and a methanol feed (100% methanol plus 12 ml/L PTM_1) was initiated at 20 ml/hr. With the initiation of the methanol feed, the set point of the pH controller was adjusted to 2.8. The pH then gradually decreased to the set point value as a result of cellular metabolism. After 4 hours of methanol feeding, the methanol feed rate was increased to 60 ml/hour and maintained at this rate for a total of approximately 72 hours, at which point the vessel was harvested.

ii. **IGF-1 expression levels of *PEP4* and *PEP4* IGF-1-expressing strains**

One of the several forms of IGF-1 produced in fermentations of recombinant IGF-1-secreting strains of *P. pastoris* is a nicked species consisting of two or more fragments of the IGF-1 molecule held together by

disulfide bonds. The fragments are generated by proteolytic cleavage of one or more peptide bonds of the amino acid backbone of the IGF-1 molecule. Although nicked and intact IGF-1 molecules are indistinguishable on the basis of apparent molecular weight [under non-reducing conditions, as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)], these species can be resolved by reverse phase HPLC under non-reducing conditions and by SDS-PAGE under reducing conditions (i.e., in the presence of a reducing agent such as dithiothreitol). Reduction of the disulfide bonds holding the fragments of nicked IGF-1 together results in liberation of the individual proteolytically generated IGF-1 fragments which have smaller molecular weights than the intact molecule.

Quantitation of IGF-1 expression levels

The yields of nicked and authentic (intact, correctly folded, monomeric) IGF-1 in the cell-free broth were determined by quantitative reverse phase HPLC. The HPLC system that was used was the same as that described in Example IV, except a Vydac C4 column (0.46 x 5 cm) was employed instead of a C18 column. A 1%/minute gradient of 25-42% mobile phase B was passed through the column during a period of 17 minutes at a flow rate of 1 ml/minute to elute samples from the column. The detector was set at 0.05 absorbance units full scale (AUFS), and a wavelength of 215 nm was used for maximum sensitivity.

To distinguish the authentic and nicked IGF-1 species in *P. pastoris* broth by HPLC, it was necessary to clean-up the broth by removing some endogenous *P. pastoris* contaminants from the broth prior to loading broth samples onto the HPLC column. This was accomplished by passing the broth through a sulphopropyl-based cation exchange resin contained in a 0.25 ml column. The resin was first washed with 2 ml of 0.2 M

acetic acid, then equilibrated with 2 ml of 0.02 M acetic acid. A volume of crude cell-free broth (1 ml) was loaded onto the column which was then washed with 1 ml of 0.02 M acetic acid. The IGF-1 was eluted with 2 ml of 0.02 M sodium acetate, pH 5.5, plus 1 M NaCl. The first 1 ml of eluate contained 75-80% of the total IGF-1 and was usually the only elution volume collected. The column was then regenerated by washing with 2 ml of 100% methanol and thereby available for re-use.

In order to quantitate the levels of Pichia-produced IGF-1, known amounts of standard IGF-1 (Amgen, Thousand Oaks, CA) were injected into the HPLC column and the area under the corresponding peaks in the chromatograms was measured. A standard curve was generated by plotting area versus μg of IGF-1 loaded onto the HPLC column. A correlation coefficient for use in converting the area under HPLC chromatogram peaks to IGF-1 concentration was calculated from the standard curve. When the detector was set at 0.05 AUFS and a wavelength of 215 nm, the correlation coefficient was 350 units/μg of IGF-1 injected onto the column. Using this information, it was possible to determine the concentration of correctly folded, intact monomeric IGF-1 present in a cleaned-up broth sample by measuring the area under the corresponding peak on the chromatogram from HPLC analysis of the sample. This correlation coefficient was also used to estimate the approximate concentration of the nicked IGF-1 species as well. However, the absolute concentrations of the nicked species may vary depending on differences in the specific correlation coefficients of intact and nicked IGF-1.

Results of one-liter fermentations

One-liter low pH (pH 2.8) fermentations of the *PEP4* IGF-1-expressing strain consistently yielded greater amounts of total monomeric (authentic plus nicked) IGF-1

(~200-250 mg/l) than one-liter low pH fermentations of the *PEP4* IGF-1-expressing strains (~160-190 mg/L). Furthermore, the percentage of authentic IGF-1 in the broth of the *PEP4* strain was somewhat higher (77%) than that in the broth of the *PEP4* strains (65%). However, a much more dramatic difference in the monomeric IGF-1 production levels of the *PEP4* and *PEP4* strains was detected in pH 5.0 fermentations of these strains. Essentially no IGF-1 was detected in one-liter pH 5.0 fermentations of the *PEP4* IGF-1-expressing strains G+IMB204S14 and G+IMB206S1. This result indicates that the authentic IGF-1 produced in fermentations of *PEP4* strains is subjected to extensive proteolysis at pH 5.0, but to only limited proteolysis at lower pH. In contrast, one-liter pH 5.0 fermentations of the *PEP4* IGF-1-expressing strain M+IMB206S1 yielded at least 200 mg of monomeric IGF-1/l, approximately 80% of which was authentic IGF-1. The *PEP4* IGF-1-expressing strain thus appears to be significantly improved relative to the *PEP4* IGF-1-expressing strains for production of authentic IGF-1 at pH 5.0 and somewhat improved for production of authentic IGF-1 at pH 2.8.

Results of ten-liter fermentations

Ten-liter fermentations of the *PEP4* IGF-1-expressing strain of *P. pastoris* yielded greater amounts of total monomeric IGF-1 (~200 mg/l) than did ten-liter fermentations of the *PEP4* IGF-1-expressing strains (~170 mg/l).

The compositions of the total monomeric IGF-1 produced in 10-liter fermentations of the *PEP4* and *PEP4* strains also differed. Greater than 75% (164 mg/l) of the total monomeric IGF-1 in the 10-liter fermentation of the *PEP4* strain M+IMB206S1 was authentic IGF-1, whereas only about 50% (88 mg/l) of the total monomeric IGF-1 in

the 10-liter fermentation of the *PEP4* strain G+IMB204S14 was authentic IGF-1.

Furthermore, because the cell yield in the fermentation of the *PEP4* strain was ~30% less than the cell yield in the fermentation of the *PEP4* strain, the per cell yield of authentic IGF-1 was greatly enhanced in the fermentation of the *PEP4* strain. As a consequence of lower cell yield in the fermentation of the *PEP4* strain, a greater volume of cell-free broth was recovered from the fermentation of the *PEP4* strain (relative to the volume of cell-free broth recovered from the fermentation of the *PEP4* strain). This results in the recovery of higher levels of secreted IGF-1 from the fermentation of the *PEP4* strains (relative to the amount of secreted IGF-1 recovered from the fermentation of the *PEP4* strain).

The results presented above demonstrate that the *PEP4* IGF-1-expressing strain is improved, relative to the *PEP4* IGF-1-expressing strain, for production of authentic IGF-1 on a large scale.

B. Generation of an IGF-1-Expressing *PEP4*⁻ Strain by Gene Replacement

1. Construction of the *P. pastoris* gene disruption vectors pDR601 and pDR602

Vectors pDR601 and pDR602 were used in the development of *PEP4*-deficient (*PEP4*⁻) strains of *P. pastoris* by disruption of a host *PEP4* gene through replacement of the endogenous *PEP4* gene with a defective *PEP4* gene. This vector was constructed in several steps as follows (see also diagram in Figure 13).

Plasmid pEP301 (see Figure 4), consisting of pUC19 sequences and the cloned *P. pastoris PEP4* gene from pEP202, was cleaved with NcoI, and the DNA was then precipitated with ethanol, harvested, resuspended and ligated in ligation reaction mixture. This digestion and ligation effectively removed an internal portion of the

PEP4 gene contained in an ~0.5 kb NcoI fragment. After ligation, the DNA was digested with BglII to linearize any remaining parental plasmid, and the DNA was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and screened by analysis of restriction enzyme digests of colony DNA for the presence of a 0.5 kb NcoI fragment. The correct plasmid, containing the defective *PEP4* gene lacking an ~0.5 kb NcoI fragment, was named pDL321. A second plasmid, pUC19XX, was generated by cleaving pUC19 with SmaI and HincII and religating, effectively removing a portion of the polylinker containing the BamHI and XbaI sites. Plasmid pUC19XX was then cut with SacI and EcoRI and ~10 ng was ligated with ~50 ng of the SacI/EcoRI 2.2 kb fragment of pDL321, which had been gel-purified and isolated with DE81 paper. The ligation mix was used to transform MC1061 cells, and ampicillin-resistant colonies were screened by analysis of BstEII/XbaI-digested colony DNA. Plasmid showing the correct digest pattern was designated pDL322.

pDL322 was then cut with XbaI and 10 ng were ligated with 10 ng of an oligonucleotide linker of the sequence 5'-CTAGCGGCCG-3', which destroyed the XbaI site and generated a unique NotI site when ligated into the XbaI site. The ligation mix was used to transform MC1061 cells. Ampicillin-resistant colonies were screened by analysis of NotI-digested colony DNA. The correct plasmid was called pDL323.

To generate vectors pDR601 and pDR602, the *Pichia URA3* gene was inserted into pDL323 as follows. Plasmid pPU205 (see Figure 9) was digested with PvuII and AatI to liberate the *URA3* gene on an approximately 2.5 kb PvuII fragment. The digest was separated on a 0.8% agarose gel. The ~2.5 kb fragment was isolated from the gel using DE81 paper, eluted and purified. Plasmid pDL323

was linearized by cutting it with EcoRV. This linearized plasmid (~10 ng) was ligated with the *URA3*-bearing PvuII fragment of pPU205 to generate pDR601 and pDR602 (see Figures 14 and 15, respectively), depending upon the orientation of the inserted *URA3* gene.

2. Transformation of IGF-U with pDR601 and pDR602

The *URA3* IGF-1-expressing *P. pastoris* strain IGF-U (see Example VI.A.2.a.) was transformed with linear fragments of DNA derived from pDR601 and pDR602. The linear fragments contained the *URA3* gene flanked on each side with DNA coding for a portion of the *PEP4* gene. Homology between the ends of the fragments and the *PEP4* gene stimulated integration of the fragments at the *PEP4* locus resulting in a gene replacement event. Stable integration of either fragment into the host genome yielded prototrophic transformants due to the stable presence of the *URA3* gene contained in the fragments. The transformation was conducted as follows:

Linear DNA fragments (~4.0 kb in length), consisting of the *URA3* gene flanked on each side with DNA coding for a portion of the *PEP4* gene, were obtained by digesting both pDR601 and pDR602 with NotI and BstEII. The digested DNA (20 μg) was used to transform strain IGF-U using the standard spheroplast procedure. Ura^+ colonies isolated from transformants growing on regeneration medium and subcultured onto YEPD medium were screened for carboxypeptidase Y activity using the overlay procedure described in Example II. Colonies that did not develop a red color relative to control colonies were selected for analysis by Southern blot hybridization.

3. Southern blot hybridization of DNA from transformants

Genomic DNA was isolated from the selected transformants using the method of Hoffman and Winston [Gene 57: 267-272 (1987)]. Genomic DNA from each strain

was digested with BstEII. This procedure liberates a portion of the *PEP4* locus containing the region of integration of fragments of pDR601 or pDR602. Therefore, the size of this region is diagnostic for correct integration of the transforming DNA into the genome of IGF-U. The digested DNA was subjected to electrophoresis on a 0.8% agarose gel and blotted to a nitrocellulose filter. The filter was hybridized with a radiolabeled 1.4 kb XbaI/EcoRV fragment of pEP301 which contains part of the *P. pastoris PEP4* gene using standard procedures [Maniatis, T., Fritsch, E.F. and Sambrook, J. Molecular Cloning, A Laboratory Manual, pp 385-388, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA. (1982)]. Hybridization was conducted at 37°C in a solution containing 50% formamide, 6 X SSC, 5X Denhardt's solution, 20 mM Tris HCl, pH 8.0, 1 mM EDTA, 0.1% SDS and 100 μg/ml salmon sperm DNA. The filter was then washed three times in 1 x SSC, 0.1% SDS (10 min per wash) and then in 0.5 x SSC, 0.1% SDS at 65°C for 1 hr. As a comparative control, genomic DNA from *P. pastoris* strain GS115, a *PEP4* strain, was included in this analysis.

Digestion of genomic DNA from GS115 with BstEII yielded a 4.4 kb fragment that hybridized to the portion of the *PEP4* gene contained in the probe. In contrast, this probe hybridized to a 6.9 kb fragment in DNA from at least two of the transformants, IGFU2601-5 and IGFU2602-5. The larger size of the transformant *PEP4* locus as compared to the control *PEP4* locus (6.9 vs. 4.4 kb) is consistent with replacement of the host *PEP4* gene with a nonfunctional *PEP4* gene carrying the *URA3* gene within its structural region.

From these results, it was concluded that strains IGFU2601-5 and IGFU2602-5 were examples of the several *PEP4* strains generated by disruption of the *PEP4* gene of host strain IGF-U through gene replacement.

EXAMPLE VII: GENERATION OF A *PEP4*⁻ PICHIA STRAIN USING "POPOUT" VECTORS

1. Construction of *P. pastoris* gene disruption vector pDL521

Vector pDL521 was used in the development of *PEP4*-deficient (*PEP4*⁻) strains of *P. pastoris* by disruption of a host *PEP4* gene through "pop-in/pop-out" methods. In this method, a defective *PEP4* gene containing a small deletion is added to a host *PEP4* locus, and a functional *PEP4* gene is removed from the *PEP4* locus (i.e., pop-in/pop-out).

pDL521 was constructed in two steps. First, an intermediate plasmid, pDL501, was constructed by ligation of the 2.2 kb EcoRI/SacI fragment of pDL323, the 2.2 kb SacI/PstI fragment of pPU205 and the 2.7 kb EcoRI/PstI fragment of pUC19 in a three-way ligation. These three fragments were obtained as follows. pPU205, which contains the *P. pastoris URA3* gene (Figure 9), was digested with PstI and SacI. A 2.2 kb PstI-SacI fragment containing the *URA3* gene was gel isolated and purified using DE81 paper. Plasmid pDL323, harboring a defective *PEP4* gene which lacks a 0.5 kb NcoI fragment present in an intact *PEP4* gene (see Fig. 13), was digested with EcoRI and SacI. A 2.2 kb fragment containing the defective *PEP4* gene was gel isolated and purified using DE81 paper. pUC19 was digested with EcoRI and PstI. The three fragments (0.02 μg of the EcoRI/PstI-digested pUC19, 0.02 μg of the 2.2 kb PstI/SacI fragment of pPU205 and 0.02 μg of the 2.2 kb EcoRI/SacI fragment of pDL323) were ligated in a three-way ligation. The ligation mix was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were screened by analysis of NcoI-digested colony DNA. Plasmid containing the correctly ligated fragments was called pDL501. pDL501 was then cut with SacI, treated with calf alkaline phosphatase and 0.02 μg were ligated with 0.02 μg of a 1.9 kb SacI

fragment isolated from SacI-digested pEP202 and purified using DE81 paper. This added more *PEP4* flanking sequence to the 3' end of the defective *PEP4* gene in pDL501 and ensured a greater amount of homologous sequence for recombination with the endogenous *PEP4* gene during transformation of *P. pastoris* host IGF-U. The ligation mix was used to transform *E. coli* strain MC1061. DNA from ampicillin-resistant colonies was digested with BglII and SpeI and screened for the presence of the diagnostic 0.8 kb fragment indicative of the presence of the added SacI fragment from pEP202. Correct plasmid was called pDL521 (see Figure 16).

2. **Transformation of GS4-2 with pDL521**

a. **Generation of GS4-2**

A *URA3* strain of *P. pastoris* was required as a host in the generation of a *PEP4* strain by the pop-out process. A *URA3* strain was developed by direct plating of 10^6 cells of the general *HIS4 P. pastoris* host strain GS115 in 5-fluoroorotic acid medium supplemented with uracil (0.67% yeast nitrogen base, 2% agar, 2% glucose, 750 ng 5-FOA/l and 48 mg uracil/l). After one week of incubation at 30°C, a colony growing on the plate was isolated. This His^-Ura^- strain was named GS4-2.

b. **Transformation of GS4-2 and generation of a *PEP4*⁻ strain**

Plasmid pDL521 was linearized by digestion with NotI. The NotI site is located immediately 5' of the site at which sequence had been deleted from the *PEP4* gene to make it defective. The ends of the NotI fragment are homologous to sequences in the endogenous *PEP4* gene of GS4-2, which promotes integration of the fragment by homologous recombination at the *PEP4* locus.

The His^- Ura^- strain GS4-2 was transformed according to the spheroplast method with 20 μg of pDL521 which had been linearized by digestion with NotI. Transformants were selected by their ability to grow on media lacking

uracil. Twelve of these transformants were picked, genomic DNA isolated from these transformants (as described in Example VI.B.3.), cut with SalI and subjected to electrophoresis on a 0.8% agarose gel. The DNA was transferred to a nitrocellulose filter and probed with a radiolabeled 1.2 kb EcoRV/XbaI fragment of the *PEP4* gene. Two strains, GS4-2521-3 and GS4-2521-4, which appeared to have integrated pDL521 into the *PEP4* locus, based on the Southern blot hybridization pattern of genomic DNA, were chosen for further selection. These strains contained the *URA3* marker gene with an intact complete *PEP4* gene on one side and a defective *PEP4* gene (lacking ~0.5 kb of sequence) on the other side of the marker gene. This configuration of the *PEP4* locus permits recombination between the two copies of the *PEP4* gene that would result in elimination of one of the *PEP4* genes and the *URA3* gene (i.e., pop-out). Either one of the two *PEP4* genes could be evicted in this recombination event. To identify if, and when, recombination between the two *PEP4* genes occurred, strains GS4-2521-3 and GS4-2521-4 were plated onto YPD medium containing 5-FOA in a serial 10-fold dilution manner. Only Ura$^-$ strains grow in the presence of 5-FOA, and thus growth in such medium indicates the occurrence of the desired recombination event. Strains able to grow on 5-FOA-containing medium were uracil auxotrophs generated by recombination between the two copies of the *PEP4* gene. Ura$^-$ colonies appeared on the 5-FOA-containing plate after 1 week of culture at 30°C: 10 of these colonies were derived from GS4-2521-3, and 14 of these colonies were derived from GS4-2521-4.

3. **Characterization of transformants**

Fourteen of the Ura$^-$ transformant colonies were purified, genomic DNA was prepared from each, digested with EcoRI and EcoRV, subjected to electrophoresis on a

0.8% agarose gel, blotted to nitrocellulose and hybridized with a radiolabeled 1.2 kb XbaI/EcoRV fragment of the *P. pastoris PEP4* gene. DNA from 7 of the 14 isolates analyzed in this way had a hybridization profile consistent with a *PEP4* locus consisting of only a defective *PEP4* gene lacking ~0.5 kb of sequence present in an intact *PEP4* gene. Two of these strains are GS4-2521-3/7 and GS4-2521-4/1.

EXAMPLE VIII: CLONING OF A PORTION OF THE *PRB-1* GENE OF *P. PASTORIS*

The proteinase B gene, *PRB-1*, encodes a vacuolar serine endoprotease in *S. cerevisiae* [Moehle et al., Mol. Cell Bio. 7: 4390-4399 (1987)]. A portion of the equivalent gene was cloned from *P. pastoris* using polymerase chain reaction (PCR) gene amplification techniques [see, for example, Gould et al., in Proc. Natl. Acad. Sci. USA 86: 1934-1938 (1989)]. Degenerate oligonucleotides, which had homology to sequences of the *PRB-1* gene that encode regions of the proteinase B protein which are conserved across species (Moehle et al. supra.)) were synthesized for use as primers in the PCR amplification of *P. pastoris PRB-1* DNA. The oligonucleotides had the following sequences:

Oligonucleotide 1:

```
                          A       A       A   G
5'- GATAGAATTCTGCAG GGT AAT GGT CAT GGT ACT CAT TGT GC-3'
                        C   C   C   C   C   C   C   C
                                                A
```

Oligonucleotide 2:

```
                  GA  A           A       A
5'- GATCGCATGC AAT CCT GCA ACA TGT GGA GAT GCC AT-3'
                  G   G   G   G   G   G CTG
```

To facilitate subcloning of the amplified DNA fragments into shuttle plasmids, each oligonucleotide also contained one or more restriction endonuclease recognition sites on its 5' end: a SphI site on oligonucleotide 2 and both PstI and EcoRI sites on oligonucleotide 1.

The PCR reaction medium consisted of 100 ng of *P. pastoris* (Strain NRRL Y-11430) genomic DNA in 2 μl of T.E. (10 mM Tris·HCl, 1 mM EDTA), 10 μl of oligonucleotide 1 and 10 μl of oligonucleotide 2, 16 μl of a 1.25 mM solution of dGTP, dCTP, dATP, and dTTP, 10 μl of 10x buffer (500 mM KCl, 100 mM Tris·HCl, pH 8.3, 15 mM $MgCl_2$), 0.1% gelatin, 70 μl of water and 0.5 μl of 5 units/μl Taq DNA polymerase. The solution was heated at 94°C for 2 minutes. The PCR cycling reaction, which was repeated 31 times, included denaturating for 2 minutes at 96°C, annealing for 1 minute at 50°C and polymerizing for 3.5 minutes at 72°C.

The product of this PCR was subjected to electrophoresis on an agarose gel, and a fragment of the size predicted (~500 bp) for the product of amplification of the *PRB-1* gene between positions corresponding to oligonucleotides 1 and 2 was isolated on DE81 paper, and digested with EcoRI and SphI, subjected to electrophoresis on an agarose gel. The 500 bp fragment was isolated using DE81 paper and was ligated into 10 ng of pUC19, which had been linearized by cutting with EcoRI and SphI in the polylinker. The ligation mix was used to transform *E. coli* MC1061 cells. Restriction enzyme-digested plasmid DNA from ampicillin-resistant transformants was analyzed for the presence of the correct 500 bp EcoRI-SphI fragment. One colony contained the correct plasmid, designated pPRBPP. A restriction map of the *Pichia* portion of this plasmid is set forth in Figure 17.

The sequence of the cloned portion of the *P. pastoris* PRB-1 gene contained in pPRBPP was generated using the Sanger dideoxy method (see Sanger et al., supra) and is shown in Sequence ID No. 5. This sequence of the *P. pastoris PRB-1* gene has approximately 74% homology to the sequence of the *S. cerevisiae PRB-1* gene.

EXAMPLE IX: DEVELOPMENT OF A *PRB-1* STRAIN OF *P. PASTORIS*

Plasmid pDR911 was constructed for use in developing *PRB-1* strains of *P. pastoris*. This vector contains an internal portion of the *P. pastoris PRB-1* gene, which, when used to transform *PRB-1* strains of *P. pastoris*, integrates into the host genome at the *PRB-1* locus to generate two incomplete and non-functional copies of the *PRB-1* gene. Vector pDR911 also contains a complete functional *P. pastoris URA3* gene for use as a selectable marker in *URA3* host strains of *P. pastoris*.

A. Construction of pDR911

The *PRB-1* gene fragment of *P. pastoris* in pPRBPP was isolated by restriction digestion of pPRBPP with PstI and SphI. The reaction mixture was loaded onto a 0.8% agarose gel and the 0.5 kb fragment was purified with DE81 paper.

This 0.5 kb fragment was ligated into a linear form of plasmid pPU203, a *P. pastoris URA3*-containing pUC-based plasmid (see Figure 8). Plasmid pPU203 was linearized by cleavage with SphI and PstI, and ~10 ng was ligated with ~100 ng of the *Pichia* DNA fragment. The ligation mixture was used to transform *E. coli* strain MC1061 to ampicillin resistance. Ampicillin-resistant colonies were screened by analysis of PstI/SphI-digested colony DNA for the diagnostic fragment. Correct plasmid was named pDR911 (see Figure 18).

B. Transformation of GS4-2 with pDR911

To generate *PRB-1* strains of *P. pastoris*, one could transform GS4-2 by standard spheroplast transformation with pDR911 that had been linearized by digestion with BglII. Southern blot hybridization of DNA from Ura^+ transformants would enable confirmation of *PRB-1* strains created by disruption of the *PRB-1* locus. Proteinase B activity assays [see, for example, Jones et al., in Genetics 102: 665-677 (1982)] of transformants would

further confirm the proteinase B deficiency of the strains.

C. Development of a prb-1, pep4 strain of *P. pastoris*

The PRB-1 gene of the pep4, ura3, his4 strain of *P. pastoris* GS4-2521-4-5, which is an isolate of GS4-2521-4 (see Example VII), was disrupted by transformation with the vector pDR911, which had been linearized by cleavage with BglII. Transformants exhibiting the Ura^{+3} phenotype were selected and analyzed by Southern blot hybridization. A selected transformant exhibiting the expected hybridization band pattern was designated MG18. This strain was used as a host for expression of IGF-1. The IGF-1 expressing strain was designated C+IGF816S1.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: GENES WHICH INFLUENCE PICHIA PROTEOLYTIC ACTIVITY, AND USES THEREFOR

(iii) NUMBER OF SEQUENCES: 6

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2032 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 239..1468

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 239..1468

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GAATTCATAA TGGTGAGATT AGGTAATCGT CCGGAATAGG AATAGTGGTT TGGGGCGATT      60

AATCGCACCT GCCTTATATG GTAAGTACCT TGACCGATAA GGTGGCAACT ATTTAGAACA     120

AAGCAAGCCA CCTTTCTTTA TCTGTAACTC TGTCGAAGCA AGCATCTTTA CTAGAGAACA     180

TCTAAACCAT TTTACATTCT AGAGTTCCAT TTCTCAATTA CTGATAATCA ATTTAAAG       238

ATG ATA TTT GAC GGT ACT ACG ATG TCA ATT GCC ATT GGT TTG CTC TCT       286
Met Ile Phe Asp Gly Thr Thr Met Ser Ile Ala Ile Gly Leu Leu Ser
  1               5                  10                  15

ACT CTA GGT ATT GGT GCT GAA GCC AAA GTT CAT TCT GCT AAG ATA CAC       334
Thr Leu Gly Ile Gly Ala Glu Ala Lys Val His Ser Ala Lys Ile His
             20                  25                  30

AAG CAT CCA GTC TCA GAA ACT TTA AAA GAG GCC AAT TTT GGG CAG TAT       382
Lys His Pro Val Ser Glu Thr Leu Lys Glu Ala Asn Phe Gly Gln Tyr
         35                  40                  45

GTC TCT GCT CTG GAA CAT AAA TAT GTT TCT CTG TTC AAC GAA CAA AAT       430
Val Ser Ala Leu Glu His Lys Tyr Val Ser Leu Phe Asn Glu Gln Asn
     50                  55                  60

GCT TTG TCC AAG TCG AAT TTT ATG TCT CAG CAA GAT GGT TTT GCC GTT       478
Ala Leu Ser Lys Ser Asn Phe Met Ser Gln Gln Asp Gly Phe Ala Val
 65                  70                  75                  80

GAA GCT TCG CAT GAT GCT CCA CTT ACA AAC TAT CTT AAC GCT CAG TAT       526
Glu Ala Ser His Asp Ala Pro Leu Thr Asn Tyr Leu Asn Ala Gln Tyr
                 85                  90                  95

TTT ACT GAG GTA TCA TTA GGT ACC CCT CCA CAA TCG TTC AAG GTG ATT       574
Phe Thr Glu Val Ser Leu Gly Thr Pro Pro Gln Ser Phe Lys Val Ile
            100                 105                 110
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CTT GAC ACA GGA TCC TCC AAT TTA TGG GTT CCT AGC AAA GAT TGT GGA      622
Leu Asp Thr Gly Ser Ser Asn Leu Trp Val Pro Ser Lys Asp Cys Gly
        115                 120                 125

TCA TTA GCT TGC TTC TTG CAT GCT AAG TAT GAC CAT GAT GAG TCT TCT      670
Ser Leu Ala Cys Phe Leu His Ala Lys Tyr Asp His Asp Glu Ser Ser
    130                 135                 140

ACT TAT AAG AAG AAT GGT AGT AGC TTT GAA ATT AGG TAT GGA TCC GGT      718
Thr Tyr Lys Lys Asn Gly Ser Ser Phe Glu Ile Arg Tyr Gly Ser Gly
145                 150                 155                 160

TCC ATG GAA GGG TAT GTT TCT CAG GAT GTG TTG CAA ATT GGG GAT TTG      766
Ser Met Glu Gly Tyr Val Ser Gln Asp Val Leu Gln Ile Gly Asp Leu
                165                 170                 175

ACC ATT CCC AAA GTT GAT TTT GCT GAG GCC ACA TCG GAG CCG GGG TTG      814
Thr Ile Pro Lys Val Asp Phe Ala Glu Ala Thr Ser Glu Pro Gly Leu
            180                 185                 190

GCC TTC GCT TTT GGC AAA TTT GAC GGA ATT TTG GGG CTT GCT TAT GAT      862
Ala Phe Ala Phe Gly Lys Phe Asp Gly Ile Leu Gly Leu Ala Tyr Asp
        195                 200                 205

TCA ATA TCA GTA AAT AAG ATT GTT CCT CCA ATT TAC AAG GCT TTG GAA      910
Ser Ile Ser Val Asn Lys Ile Val Pro Pro Ile Tyr Lys Ala Leu Glu
    210                 215                 220

TTA GAT CTC CTT GAC GAA CCA AAA TTT GCC TTC TAC TTG GGG GAT ACG      958
Leu Asp Leu Leu Asp Glu Pro Lys Phe Ala Phe Tyr Leu Gly Asp Thr
225                 230                 235                 240

GAC AAA GAT GAA TCC GAT GGC GGT TTG GCC ACA TTT GGT GGT GTG GAC      1006
Asp Lys Asp Glu Ser Asp Gly Gly Leu Ala Thr Phe Gly Gly Val Asp
                245                 250                 255

AAA TCT AAG TAT GAA GGA AAG ATC ACC TGG TTG CCT GTC AGA AGA AAG      1054
Lys Ser Lys Tyr Glu Gly Lys Ile Thr Trp Leu Pro Val Arg Arg Lys
            260                 265                 270

GCT TAC TGG GAG GTC TCT TTT GAT GGT GTA GGT TTG GGA TCC GAA TAT      1102
Ala Tyr Trp Glu Val Ser Phe Asp Gly Val Gly Leu Gly Ser Glu Tyr
        275                 280                 285

GCT GAA TTG CAA AAA ACT GGT GCA GCC ATC GAC ACT GGA ACC TCA TTG      1150
Ala Glu Leu Gln Lys Thr Gly Ala Ala Ile Asp Thr Gly Thr Ser Leu
    290                 295                 300
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ATT GCT TTG CCC AGT GGC CTA GCT GAA ATT CTC AAT GCA GAA ATT GGT      1198
Ile Ala Leu Pro Ser Gly Leu Ala Glu Ile Leu Asn Ala Glu Ile Gly
305                 310                 315                 320

GCT ACC AAG GGT TGG TCT GGT CAA TAC GCT GTG GAC TGT GAC ACT AGA      1246
Ala Thr Lys Gly Trp Ser Gly Gln Tyr Ala Val Asp Cys Asp Thr Arg
                325                 330                 335

GAC TCT TTG CCA GAC TTA ACT TTA ACC TTC GCC GGT TAC AAC TTT ACC      1294
Asp Ser Leu Pro Asp Leu Thr Leu Thr Phe Ala Gly Tyr Asn Phe Thr
            340                 345                 350

ATT ACT CCA TAT GAC TAT ACT TTG GAG GTT TCT GGG TCA TGT ATT AGT      1342
Ile Thr Pro Tyr Asp Tyr Thr Leu Glu Val Ser Gly Ser Cys Ile Ser
        355                 360                 365

GCT TTC ACC CCC ATG GAC TTT CCT GAA CCA ATA GGT CCT TTG GCA ATC      1390
Ala Phe Thr Pro Met Asp Phe Pro Glu Pro Ile Gly Pro Leu Ala Ile
    370                 375                 380

ATT GGT GAC TCG TTC TTG AGA AAA TAT TAC TCA GTT TAT GAC CTA GGC      1438
Ile Gly Asp Ser Phe Leu Arg Lys Tyr Tyr Ser Val Tyr Asp Leu Gly
385                 390                 395                 400

AAA GAT GCA GTA GGT TTA GCC AAG TCT ATT TAGGCAAGAA TAAAAGTTGC        1488
Lys Asp Ala Val Gly Leu Ala Lys Ser Ile
                405                 410

TCAGCTGAAC TTATTTGGTT ACTTATCAGG TAGTGAAGAT GTAGAGAATA TATGTTTAGG    1548

TATTTTTTTT TAGTTTTTCT CCTATAACTC ATCTTCAGTA CGTGATTGCT TGTCAGCTAC    1608

CTTGACAGGG GCGCATAAGT GATATCGTGT ACTGCTCAAT CAAGATTTGC CTGCTCCATT    1668

GATAAGGGTA TAAGAGACCC ACCTGCTCCT CTTTAAAATT CTCTCTTAAC TGTTGTGAAA    1728

ATCATCTTCG AAGCAAATTC GAGTTTAAAT CTATGCGGTT GGTAACTAAA GGTATGTCAT    1788

GGTGGTATAT AGTTTTTCAT TTTACCTTTT ACTAATCAGT TTTACAGAAG AGGAACGTCT    1848

TTCTCAAGAT CGAAATAGGA CTAAATACTG GAGACGATGG GGTCCTTATT TGGGTGAAAG    1908

GCAGTGGGCT ACAGTAAGGG AAGACTATTC CGATGATGGA GATGCTTGGT CTGCTTTTCC    1968

TTTTGAGCAA TCTCATTTGA GAACTTATCG CTGGGGAGAG GATGGACTAG CTGGAGTCTC    2028

AGAC                                                                 2032
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 410 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ile Phe Asp Gly Thr Thr Met Ser Ile Ala Ile Gly Leu Leu Ser
 1               5                  10                  15

Thr Leu Gly Ile Gly Ala Glu Ala Lys Val His Ser Ala Lys Ile His
            20                  25                  30

Lys His Pro Val Ser Glu Thr Leu Lys Glu Ala Asn Phe Gly Gln Tyr
        35                  40                  45

Val Ser Ala Leu Glu His Lys Tyr Val Ser Leu Phe Asn Glu Gln Asn
    50                  55                  60

Ala Leu Ser Lys Ser Asn Phe Met Ser Gln Gln Asp Gly Phe Ala Val
65                  70                  75                  80

Glu Ala Ser His Asp Ala Pro Leu Thr Asn Tyr Leu Asn Ala Gln Tyr
                85                  90                  95

Phe Thr Glu Val Ser Leu Gly Thr Pro Pro Gln Ser Phe Lys Val Ile
            100                 105                 110

Leu Asp Thr Gly Ser Ser Asn Leu Trp Val Pro Ser Lys Asp Cys Gly
        115                 120                 125

Ser Leu Ala Cys Phe Leu His Ala Lys Tyr Asp His Asp Glu Ser Ser
    130                 135                 140

Thr Tyr Lys Lys Asn Gly Ser Ser Phe Glu Ile Arg Tyr Gly Ser Gly
145                 150                 155                 160

Ser Met Glu Gly Tyr Val Ser Gln Asp Val Leu Gln Ile Gly Asp Leu
                165                 170                 175

Thr Ile Pro Lys Val Asp Phe Ala Glu Ala Thr Ser Glu Pro Gly Leu
            180                 185                 190

Ala Phe Ala Phe Gly Lys Phe Asp Gly Ile Leu Gly Leu Ala Tyr Asp
        195                 200                 205
```

```
Ser Ile Ser Val Asn Lys Ile Val Pro Pro Ile Tyr Lys Ala Leu Glu
    210                 215                 220

Leu Asp Leu Leu Asp Glu Pro Lys Phe Ala Phe Tyr Leu Gly Asp Thr
225                 230                 235                 240

Asp Lys Asp Glu Ser Asp Gly Gly Leu Ala Thr Phe Gly Gly Val Asp
                245                 250                 255

Lys Ser Lys Tyr Glu Gly Lys Ile Thr Trp Leu Pro Val Arg Arg Lys
            260                 265                 270

Ala Tyr Trp Glu Val Ser Phe Asp Gly Val Gly Leu Gly Ser Glu Tyr
        275                 280                 285

Ala Glu Leu Gln Lys Thr Gly Ala Ala Ile Asp Thr Gly Thr Ser Leu
    290                 295                 300

Ile Ala Leu Pro Ser Gly Leu Ala Glu Ile Leu Asn Ala Glu Ile Gly
305                 310                 315                 320

Ala Thr Lys Gly Trp Ser Gly Gln Tyr Ala Val Asp Cys Asp Thr Arg
                325                 330                 335

Asp Ser Leu Pro Asp Leu Thr Leu Thr Phe Ala Gly Tyr Asn Phe Thr
            340                 345                 350

Ile Thr Pro Tyr Asp Tyr Thr Leu Glu Val Ser Gly Ser Cys Ile Ser
        355                 360                 365

Ala Phe Thr Pro Met Asp Phe Pro Glu Pro Ile Gly Pro Leu Ala Ile
    370                 375                 380

Ile Gly Asp Ser Phe Leu Arg Lys Tyr Tyr Ser Val Tyr Asp Leu Gly
385                 390                 395                 400

Lys Asp Ala Val Gly Leu Ala Lys Ser Ile
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```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2688 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 643..1431

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 643..1431

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CTGCAGAAAT GGGGAGATAA CCACCTTTGA CGAATTGACT AAAGTTCTAC AGATCATGTT      60

TACAAATGCC ATCATCTATA ACGATGAAGA CAGTGATGTT TCGAAGCTAA CGATTGAAAT     120

GATGGAAGAA ACTACTAAGA TTATAGAGCT GTTCAGAGAA AGTCTGGATT AGTCCTGGAC     180

AATGAACTTT ATGTACAAAA ATATGGGGTT AACGTCTTAG CTGTTGCATC ATAAGTTGGT     240

TTTGTTCTTG GAAACGTTGA CCAACTCTCT CACTGTGCTT GAGGAACTTT TCTGCACACT     300

TGTTGATGCA GCCTTCCTCC TTAGAAGTCA ACTTGTTAGA TGTAAAATCA TTGACACAGT     360

CTGTAAAACA TTTGCTAACC AAATCGGAGT AAAGACGCAT GAAGTCTTTC ATTTGTTTTT     420

GTTCAACGAG TTTCTGGAAC TCTTGTTGTT CTTTAGCGTT CAATGCGTCC ATTTTGTGAT     480

GTACTTGGTT GGGGTAGAGT TAGCACTTGC TCTCTCTGTT ACCAGTTTTT GTCAAGATTG     540

AAGAAAAAAG TTTTTTGGAC GGTACACGTC GCACCTATCC TTCGCATTGA TCCACTCTAA     600

TGAGTTAACA TCAACCTGAT CAAAGGGATA GATACCTAGA CA ATG GCT CGC AGT        654
                                                 Met Ala Arg Ser
                                                   1

TAT GCC GAG AGA GCA AAT ACT CAT CAA TCA CCT GTG GCA CGA CGA CTG       702
Tyr Ala Glu Arg Ala Asn Thr His Gln Ser Pro Val Ala Arg Arg Leu
  5                  10                  15                  20

TTT GCG CTT ATG GAA CAG AAA CAG AGT AAC CTA TGC GCA TCA GTC GAC      750
Phe Ala Leu Met Glu Gln Lys Gln Ser Asn Leu Cys Ala Ser Val Asp
                 25                  30                  35

GTG AGA ACA ACT AAA GAA TTA TTG GAG CTT CTA GAT AAA TTG GGC CCA      798
Val Arg Thr Thr Lys Glu Leu Leu Glu Leu Leu Asp Lys Leu Gly Pro
             40                  45                  50

TTT ATC TGT TTG GCC AAG ACT CAT ATC GAC ATA ATT GAT GAC TTC ACG      846
Phe Ile Cys Leu Ala Lys Thr His Ile Asp Ile Ile Asp Asp Phe Thr
         55                  60                  65
```

```
TAT GAT GGA ACT ATT CTG CCT TTA TTG GAA CTA TCA AAG AAA CAC AAG        894
Tyr Asp Gly Thr Ile Leu Pro Leu Leu Glu Leu Ser Lys Lys His Lys
     70                  75                  80

TTT TTA ATT TTT GAG GAC AGA AAG TTT GCT GAT ATA GGC AAC ACT GTC        942
Phe Leu Ile Phe Glu Asp Arg Lys Phe Ala Asp Ile Gly Asn Thr Val
 85                  90                  95                 100

AAG CAT CAA TAT CAA GGA GGT GTC TAC AAG ATT GCA CAA TGG GCA GAT        990
Lys His Gln Tyr Gln Gly Gly Val Tyr Lys Ile Ala Gln Trp Ala Asp
                105                 110                 115

ATT ACA AAT GCT CAT GGT GTC ATT GGT AGT GGA ATT GTA AAG GGT CTA       1038
Ile Thr Asn Ala His Gly Val Ile Gly Ser Gly Ile Val Lys Gly Leu
            120                 125                 130

AAG GAG GCA GCC ACT GAG ACA ACA GAT CAA CCA AGG GGA CTA TTG ATG       1086
Lys Glu Ala Ala Thr Glu Thr Thr Asp Gln Pro Arg Gly Leu Leu Met
        135                 140                 145

TTG GCT GAA CTG TCG TCA AAG GGA TCA ATT GCC CAT GGT AAG TAC ACC       1134
Leu Ala Glu Leu Ser Ser Lys Gly Ser Ile Ala His Gly Lys Tyr Thr
    150                 155                 160

GAA GAA ACT GTA GAA ATT GCA AAA TCA GAC AAG GAA TTC GTC ATT GGG       1182
Glu Glu Thr Val Glu Ile Ala Lys Ser Asp Lys Glu Phe Val Ile Gly
165                 170                 175                 180

TTT ATT GCT CAA AAT TCT ATG GGA GGA CAA GAT GAA GGG TTC GAT TGG       1230
Phe Ile Ala Gln Asn Ser Met Gly Gly Gln Asp Glu Gly Phe Asp Trp
                185                 190                 195

ATT ATT ATG ACA CCA GGT GTT GGT TTG GAT GAC ACT GGT GAT GCT CTA       1278
Ile Ile Met Thr Pro Gly Val Gly Leu Asp Asp Thr Gly Asp Ala Leu
            200                 205                 210

GGC CAA CAA TAT CGA ACA GTG AGT CAA GTA TTT TCC ACT GGC ACT GAC       1326
Gly Gln Gln Tyr Arg Thr Val Ser Gln Val Phe Ser Thr Gly Thr Asp
        215                 220                 225

ATC ATA ATC GTA GGT CGT GGT TTG TTT GGC AAG GGC AGA GAT CCC TTA       1374
Ile Ile Ile Val Gly Arg Gly Leu Phe Gly Lys Gly Arg Asp Pro Leu
    230                 235                 240

AAA GAA GGT GAA CGG TAT AGA AAA GCT GGG TGG GAA GCT TAC CAA AAT       1422
Lys Glu Gly Glu Arg Tyr Arg Lys Ala Gly Trp Glu Ala Tyr Gln Asn
245                 250                 255                 260

ATT CTG AGG TAAATTACAA GTATGTACAG GGGATCAATT GTTTCGGGCG               1471
Ile Leu Arg
```

ATTCAACTGA ATCGATCTTC AATTTCATCG CTCAATTTTT GACGCAGTAT TTCAAACACC 1531

AGAAGCCCCA CGGATGTTGC TGGAATGGTA GTTAACGCAT TCCTAACGAA CCCTTTATAA 1591

AACCAGCGGG TCCAAGATAG TTTAGACTTC TCATGTAAGC TCACCAACTG GTGGAATGTA 1651

TCTAAGTATG ATCGGTAATA TAGACGGAAT TTACTTTTCT TATCCCAGGA GTTCTCGTTG 1711

AAAATATCCA ACGCTTCCAA CCTTGCTAAA TGTATTGACT GAACTTTAGA AAATGGGTAT 1771

TGAACGGCTA GTAACGAACA TGCAGCGCTA GCACCAGCCA AAAGAATAAA AGTCGTCCTC 1831

AGGATATTTT CACTTTTCGT TTTCACTGTG TCACCTTGGG GCCTTCCAAG AAGACTATTT 1891

TTCATCCTAT CAATTCTCTC CATAGTGTTC TCGGTTATCC TGTAACCTCT ATTCTTAATG 1951

GCTTCGAATG TTGTGAAATA TATAGCAAAG GATGTGCTTT CTTTGACCAG ACTCAAGGAG 2011

TAGCCAGCAA ATACCCCCCAG AAAACCACTA GTTTTTAGTT TATGAAGACC GTAAATCCAT 2071

AAGTTGTCAT TCTTGCCCCC AATAATCTCG GAGGCATTAG ATCGGGCATA TATTGCATCA 2131

ATTGGGGCAG CTACCAATGA CTGCGCAGCT CCAGCTAGAA ACCCAGCTCG AAATACATCC 2191

ACTAGTCTTG GATTTGCTAT CGATCTGCCC TCTTGACCGT CAGTATATGA CTGCAAACAT 2251

GATAAATACG TTGTGTAAAG TACAATTCCC ATCACAGAAT TGGCTACCAA TGGTGGCAGG 2311

ACCTTGTTTG GTATCAACTC CCAACCATGG GTTTTGACGG CTCGTAACAA TAGAGCTGGA 2371

TTTGAGTGGA AAATGGGCTG TAAGGTTTAC CTTTCAAATG AGCTCCAAAG AAGATGCGTA 2431

TTGGTGCCAT GTAGTCAAAA CGAGTGGGAC GAAACAGTTT GGCTGGTGTC CTCAGGTACA 2491

GTGAACTAAA TTGGACTAGA ACAGCTCTGA TCCCAGCTGT CGAAGCAGAC ACCACTTGAG 2551

TGTTTTTGTT GCTAAGAGTA GCCTTTTTAG AATCATCGTT GTCTTCCATA GGTTTCTGGA 2611

ACACAATGCC AGAGTTCATA GAGGATCAGA GGGGAATTGA GGTGTGTGTA TATGTATTTA 2671

TAGGGGTACC GAGCTCG 2688

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 263 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Ala Arg Ser Tyr Ala Glu Arg Ala Asn Thr His Gln Ser Pro Val
  1               5                  10                  15

Ala Arg Arg Leu Phe Ala Leu Met Glu Gln Lys Gln Ser Asn Leu Cys
             20                  25                  30

Ala Ser Val Asp Val Arg Thr Thr Lys Glu Leu Leu Glu Leu Leu Asp
         35                  40                  45

Lys Leu Gly Pro Phe Ile Cys Leu Ala Lys Thr His Ile Asp Ile Ile
     50                  55                  60

Asp Asp Phe Thr Tyr Asp Gly Thr Ile Leu Pro Leu Leu Glu Leu Ser
 65                  70                  75                  80

Lys Lys His Lys Phe Leu Ile Phe Glu Asp Arg Lys Phe Ala Asp Ile
                 85                  90                  95

Gly Asn Thr Val Lys His Gln Tyr Gln Gly Gly Val Tyr Lys Ile Ala
            100                 105                 110

Gln Trp Ala Asp Ile Thr Asn Ala His Gly Val Ile Gly Ser Gly Ile
        115                 120                 125

Val Lys Gly Leu Lys Glu Ala Ala Thr Glu Thr Thr Asp Gln Pro Arg
    130                 135                 140

Gly Leu Leu Met Leu Ala Glu Leu Ser Ser Lys Gly Ser Ile Ala His
145                 150                 155                 160

Gly Lys Tyr Thr Glu Glu Thr Val Glu Ile Ala Lys Ser Asp Lys Glu
                165                 170                 175

Phe Val Ile Gly Phe Ile Ala Gln Asn Ser Met Gly Gly Gln Asp Glu
            180                 185                 190

Gly Phe Asp Trp Ile Ile Met Thr Pro Gly Val Gly Leu Asp Asp Thr
        195                 200                 205
```

```
Gly Asp Ala Leu Gly Gln Gln Tyr Arg Thr Val Ser Gln Val Phe Ser
    210                 215                 220

Thr Gly Thr Asp Ile Ile Ile Val Gly Arg Gly Leu Phe Gly Lys Gly
225                 230                 235                 240

Arg Asp Pro Leu Lys Glu Gly Glu Arg Tyr Arg Lys Ala Gly Trp Glu
                245                 250                 255

Ala Tyr Gln Asn Ile Leu Arg
            260
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 555 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..554

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 3..554

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GA ATT CTG CAG GGA AAC GGC CAC GGT ACA CAT TGT GCT GGT ACC ATT          47
   Ile Leu Gln Gly Asn Gly His Gly Thr His Cys Ala Gly Thr Ile
    1               5                  10                  15

GCT TCT GAA AGC TAC GGT GTT GCC AAG AAG GCT AAT GTT GTT GCC ATC          95
Ala Ser Glu Ser Tyr Gly Val Ala Lys Lys Ala Asn Val Val Ala Ile
                20                  25                  30

AAG GTC TTG AGA TCT AAT GGT TCT GGT TCG ATG TCA GAT GTT CTG AAG         143
Lys Val Leu Arg Ser Asn Gly Ser Gly Ser Met Ser Asp Val Leu Lys
            35                  40                  45

GGT GTT GAG TAT GCC ACC CAA TCC CAC TTG GAT GCT GTT AAA AAG GGC         191
Gly Val Glu Tyr Ala Thr Gln Ser His Leu Asp Ala Val Lys Lys Gly
        50                  55                  60
```

```
AAC AAG AAA TTT AAG GGC TCT ACC GCT AAC ATG TCA CTG GGT GGT GGT      239
Asn Lys Lys Phe Lys Gly Ser Thr Ala Asn Met Ser Leu Gly Gly Gly
     65                  70                  75

AAA TCT CCT GCT TTG GAC CTT GCA GTC AAT GCT GCT GTT AAG AAT GGT      287
Lys Ser Pro Ala Leu Asp Leu Ala Val Asn Ala Ala Val Lys Asn Gly
 80                  85                  90                  95

ATT CAC TTT GCC GTT GCA GCA GGT AAC GAA AAC CAA GAT GCT TGT AAC      335
Ile His Phe Ala Val Ala Ala Gly Asn Glu Asn Gln Asp Ala Cys Asn
                100                 105                 110

ACC TCG CCA GCA GCT GCT GAG AAT GCC ATC ACC GTC GGT GCA TCA ACC      383
Thr Ser Pro Ala Ala Ala Glu Asn Ala Ile Thr Val Gly Ala Ser Thr
            115                 120                 125

TTA TCA GAC GCT AGA GCT TAC TTT TCT AAC TAC GGT AAA TGT GTT GAC      431
Leu Ser Asp Ala Arg Ala Tyr Phe Ser Asn Tyr Gly Lys Cys Val Asp
        130                 135                 140

ATT TTC GCT CCA GGT TTA AAC ATT CTT TCT ACC TAC ACT GGT TCG GAT      479
Ile Phe Ala Pro Gly Leu Asn Ile Leu Ser Thr Tyr Thr Gly Ser Asp
    145                 150                 155

GAC GCA ACT GCT ACC TTG TCT GGT ACT TCA ATG GCC AGC CCT CAT GTT      527
Asp Ala Thr Ala Thr Leu Ser Gly Thr Ser Met Ala Ser Pro His Val
160                 165                 170                 175

GCA GGC TTG CAT GCA AGC TTG GCA CTG G                               555
Ala Gly Leu His Ala Ser Leu Ala Leu
                180
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 184 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Ile Leu Gln Gly Asn Gly His Gly Thr His Cys Ala Gly Thr Ile Ala
  1               5                  10                  15

Ser Glu Ser Tyr Gly Val Ala Lys Lys Ala Asn Val Val Ala Ile Lys
             20                  25                  30
```

Val Leu Arg Ser Asn Gly Ser Gly Ser Met Ser Asp Val Leu Lys Gly
35 40 45

Val Glu Tyr Ala Thr Gln Ser His Leu Asp Ala Val Lys Lys Gly Asn
50 55 60

Lys Lys Phe Lys Gly Ser Thr Ala Asn Met Ser Leu Gly Gly Gly Lys
65 70 75 80

Ser Pro Ala Leu Asp Leu Ala Val Asn Ala Ala Val Lys Asn Gly Ile
85 90 95

His Phe Ala Val Ala Ala Gly Asn Glu Asn Gln Asp Ala Cys Asn Thr
100 105 110

Ser Pro Ala Ala Ala Glu Asn Ala Ile Thr Val Gly Ala Ser Thr Leu
115 120 125

Ser Asp Ala Arg Ala Tyr Phe Ser Asn Tyr Gly Lys Cys Val Asp Ile
130 135 140

Phe Ala Pro Gly Leu Asn Ile Leu Ser Thr Tyr Thr Gly Ser Asp Asp
145 150 155 160

Ala Thr Ala Thr Leu Ser Gly Thr Ser Met Ala Ser Pro His Val Ala
165 170 175

Gly Leu His Ala Ser Leu Ala Leu
180

THAT WHICH IS CLAIMED IS:

1. An isolated DNA fragment obtained from a strain of the genus *Pichia*, comprising a gene encoding a protein which, directly or indirectly, influences the proteolytic activity of said strain.

2. The DNA fragment of Claim 1, wherein said protein influences the carboxypeptidase Y activity of said strain.

3. The DNA fragment of Claim 2, wherein said gene has the restriction map in FIG. 1 of the drawings.

4. The DNA fragment of Claim 2, wherein said fragment is the approximately 10.6 kbp EcoRI fragment of plasmid pEP202.

5. The DNA fragment of Claim 2, wherein said fragment is the approximately 2.7 kbp EcoRI-SacI fragment of plasmid pEP301, or portions thereof that encode a protein which influences the carboxypeptidase Y activity of said strain.

6. The DNA fragment of Claim 2, wherein said the nucleic acid sequence of said gene encodes an amino acid sequence which is substantially the same as the amino acid sequence set forth in Sequence ID No. 2.

7. The DNA fragment of Claim 2, wherein the nucleic acid sequence of said gene is substantially the same as the nucleic acid sequence set forth in Sequence ID No. 1.

8. An isolated DNA fragment containing a modified form of the gene of Claim 1, wherein said modification renders the gene incapable of producing functional product, or alters the ability of the gene product to influence the proteolytic activity of said strain.

9. The DNA fragment of Claim 8, wherein the modified form of said gene is suitable for introduction into a yeast host of the genus *Pichia* by homologous recombination, wherein homologous recombination occurs at the specific locus of said gene whose expression product influences proteolytic activity.

10. The DNA fragment of Claim 8, wherein the modified gene, in its unmodified form, encodes a protein that influences the carboxypeptidase Y activity of said strain.

11. The DNA fragment of Claim 9, wherein said gene is modified by the insertion of an auxotrophic marker gene therein.

12. A DNA fragment of Claim 11, wherein said auxotrophic marker gene is selected from the *Pichia* or *Saccharomyces HIS4* gene, the *Pichia* or *Saccharomyces ARG4* gene, or the *Pichia* or *Saccharomyces URA3* gene.

13. The DNA fragment of Claim 12, wherein said fragment is included in plasmid pDR401.

14. The DNA fragment of Claim 9, wherein said gene is modified by making deletions therefrom.

15. The DNA fragment of Claim 14, wherein said fragment is included in plasmid pDR421.

16. A method of producing strain(s) of the genus *Pichia* which are deficient in proteolytic activity, compared to host strain(s) of the same species, comprising:

contacting said host strain(s) with the DNA fragment of Claim 8 under conditions suitable for the site-directed integration of said DNA fragment into the genome of said host strain(s), wherein said site-directed integration occurs at the specific locus of said gene which encodes a protein which influences proteolytic activity.

17. The method of Claim 16, further comprising:

screening the strains obtained as a result of said contacting for the presence of strains which have reduced proteolytic activity, relative to said host strain(s), and

selecting those strains which have reduced proteolytic activity, relative to said host strain(s).

18. The method of Claim 16, wherein integration of said DNA fragment influences the proteinase A and carboxypeptidase Y activities of said host organism.

19. A strain of *Pichia* deficient in proteolytic activity produced by the method of Claim 16.

20. The strain of Claim 19, which is deficient in proteinase A and carboxypeptidase Y activities.

21. The strain of Claim 20 selected from *P. pastoris* strain p1, p2, p5, p8, p13, p16, or p20.

22. The method of Claim 16, wherein said host strain is defective in at least one auxotrophic marker

gene selected from the histidinol dehydrogenase gene, the argininosuccinate lyase gene, or the orotidine-5'-phosphate decarboxylase gene, and wherein the modified gene of said fragment is modified by the insertion therein of an intact form of the auxotrophic marker gene in which the host strain is defective.

23. A strain of *Pichia* deficient in proteolytic activity produced by the method of Claim 22.

24. A strain of Claim 23, wherein said strain is deficient in proteinase A and carboxypeptidase Y activity.

25. A strain of Claim 24 selected from *P. pastoris* strain p1, p2, p5, p8, p13, p16, or p20.

26. The method of Claim 16, wherein the gene said fragment is modified by making deletions therefrom.

27. The method of Claim 22, wherein said host strain is GS115 and said DNA fragment is the approximately 5.3 kbp SacI-EcoRI fragment of plasmid pDR401.

28. A yeast cell of the genus *Pichia* which is deficient in proteolytic activity, compared to wild-type strains of the same species.

29. The yeast cell of Claim 28, wherein said cell deficient in proteinase A and carboxypeptidase Y activities.

30. A method for the expression of proteolytically sensitive recombinant product(s), comprising:

transforming the cell of Claim 28 with DNA encoding said product and culturing said cell under conditions whereby said proteolytically sensitive product(s) is expressed.

31. The method of claim 30, wherein the cell is rendered deficient in proteolytic activity by replacement of a gene of the cell that encodes a protein which, upon expression, directly or indirectly, influences the carboxypeptidase Y activity of said host organism with a defective form of said gene modified by the insertion therein of a marker gene which complements an auxotrophic phenotype of said host strain; and wherein the marker gene is selected from the histidinol dehydrogenase gene, the argininosuccinate lyase gene, or the orotidine-5'-phosphate decarboxylase gene.

32. A method for the expression of proteolytically sensitive recombinant product(s), comprising

rendering a host strain deficient in proteolytic activity using the method of Claim 16, wherein

said host strain is defective in at least one auxotrophic marker gene; and

said host strain has been transformed with at least one DNA fragment comprising an expression cassette comprising, in the reading frame direction of transcription, the following sequences of nucleotides:

(i) a promoter region of a methanol-responsive gene of a methylotrophic yeast,

(ii) a sequence encoding a polypeptide consisting essentially of:

(a) an optional secretion signal sequence, and

(b) a proteolytically sensitive protein; and

(iii) a transcription terminator functional in a methylotrophic yeast;

wherein said sequences are operationally associated with one another for transcription of the sequences encoding said polypeptide.

33. The method of Claim 32, wherein said host strain is defective in at least two auxotrophic marker genes.

34. A method of Claim 33, wherein said auxotrophic marker genes are the *HIS4* gene, and the *URA3* gene.

35. A method of Claim 34, wherein the proteolytically sensitive product is IGF-1 and the marker gene employed for transforming said host with DNA encoding IGF-1 is the *HIS4* gene.

36. A method of Claim 35, wherein the modified gene employed to render the host deficient in proteolytic activity, in its unmodified form, encodes a protein that influences the carboxypeptidase activity of the host.

37. A method of Claim 36, wherein the modified gene is produced by deletion of a portion of the coding sequence therefrom.

38. A method of Claim 37, wherein the recombinant strain from which said recombinant product(s) is expressed is strain M+IMB206S1.

39. An isolated DNA fragment, comprising the orotidine-5'-phosphate decarboxylase gene from species of the genus *Pichia*.

40. The DNA fragment of Claim 39, wherein said gene has substantially the same restriction map as shown in FIG. 12.

41. The DNA fragment of Claim 39, wherein said gene encodes substantially the same amino acid sequence as set forth in Sequence ID No. 4.

42. A DNA fragment of Claim 39, wherein said gene has substantially the same nucleic acid sequence as set forth in Sequence ID No. 3.

43. A yeast cell of the genus *Pichia*, which is defective in the orotidine-5'-phosphate decarboxylase gene.

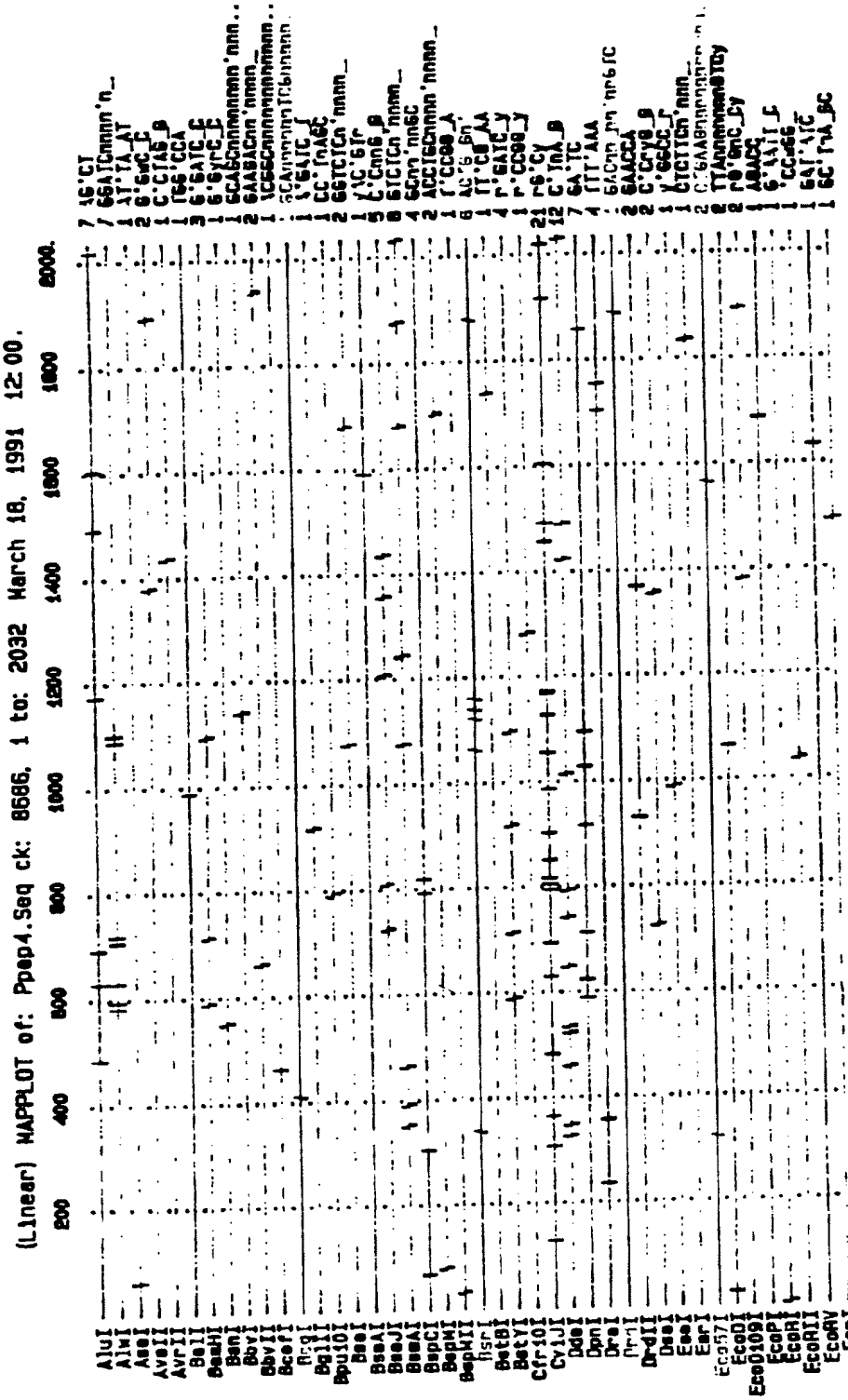
44. The yeast cell of Claim 43, wherein said yeast cell is a strain of *Pichia pastoris*.

45. The yeast cell of Claim 44, wherein said yeast cell is the strain *Pichia pastoris* GS4-2.

46. The yeast cell of Claim 43, further containing a deficiency in proteolytic activity.

47. A yeast cell of Claim 46, wherein said yeast cell is deficient in proteinase A and carboxypeptidase Y activities.

48. The yeast cell of Claim 47, wherein said yeast cell is selected from the *Pichia pastoris* strains GS4-2521-3/7 or GS4-2521-4/1.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AvrII
BalI
BamHI
BanI
BbvI
BbvII
BceFI
BglI
BglII
BpuI0I
BseI
BseAI
BseJI
BssAI
BspCI
BspMI
BspHII
BstBI
BstYI
Cfr10I
CviJI
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DpnI
DraI
DrdI
DrdII
DseI
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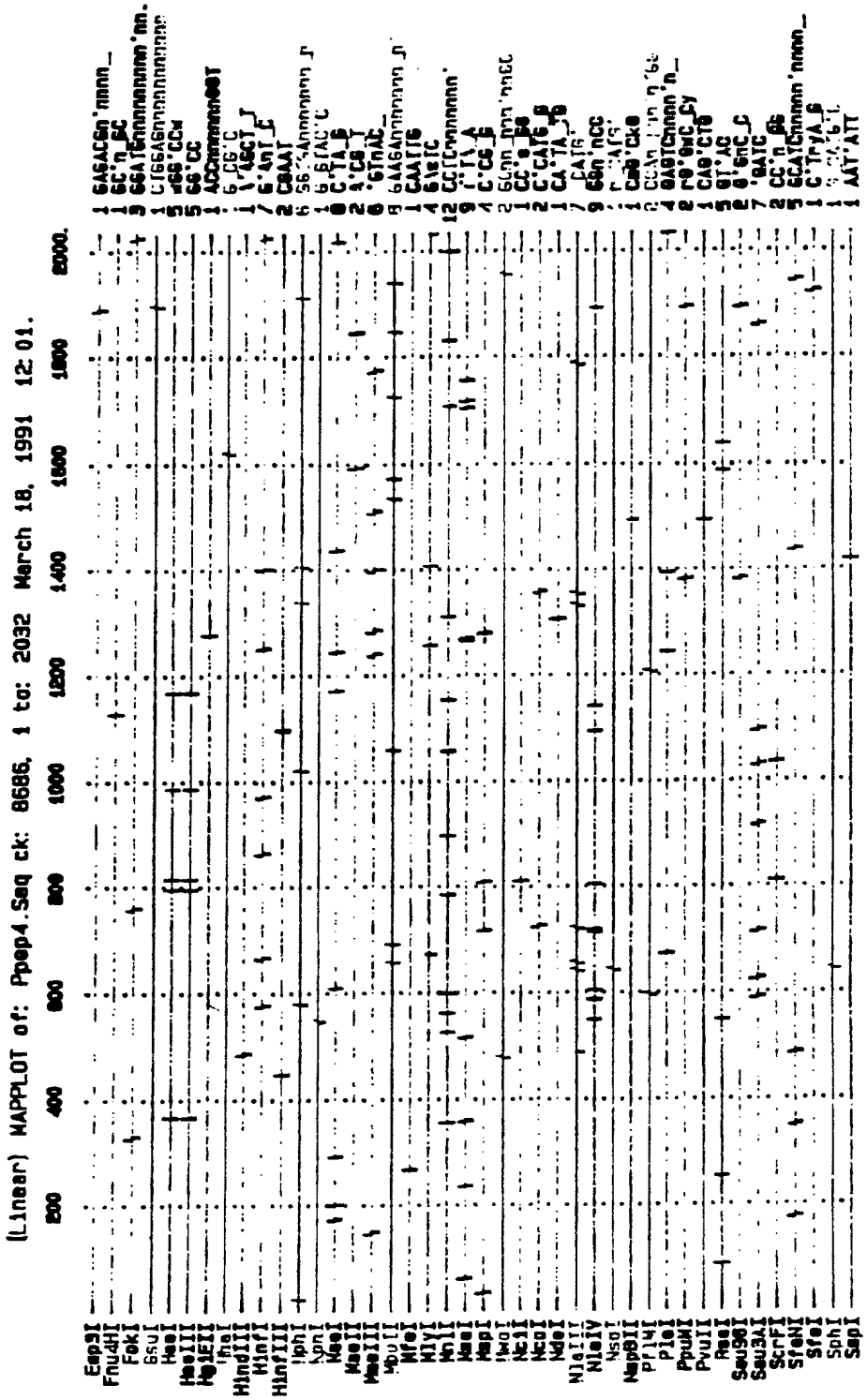
EcoRI
EcoRII
EcoRV
EspI

FIG. 1-1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FokI
BsuI
HaeI
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HgiEII
TaqI
HindIII
HinfI
HinfIII
NphI
XmnI
NaeI
MaeII
MaeIII
MboII
MfeI
MlyI
MnlI
NlaI
NlaIII
NlaIV
NsiI
NcoI
NdeI
NlaIII
NlaIV
NspBII
PflMI
PleI
PpuMI
PvuII
RsaI
Sau96I
Sau3AI
ScrFI
SfaNI
SfeI
SphI
SapI

FIG. 1-2

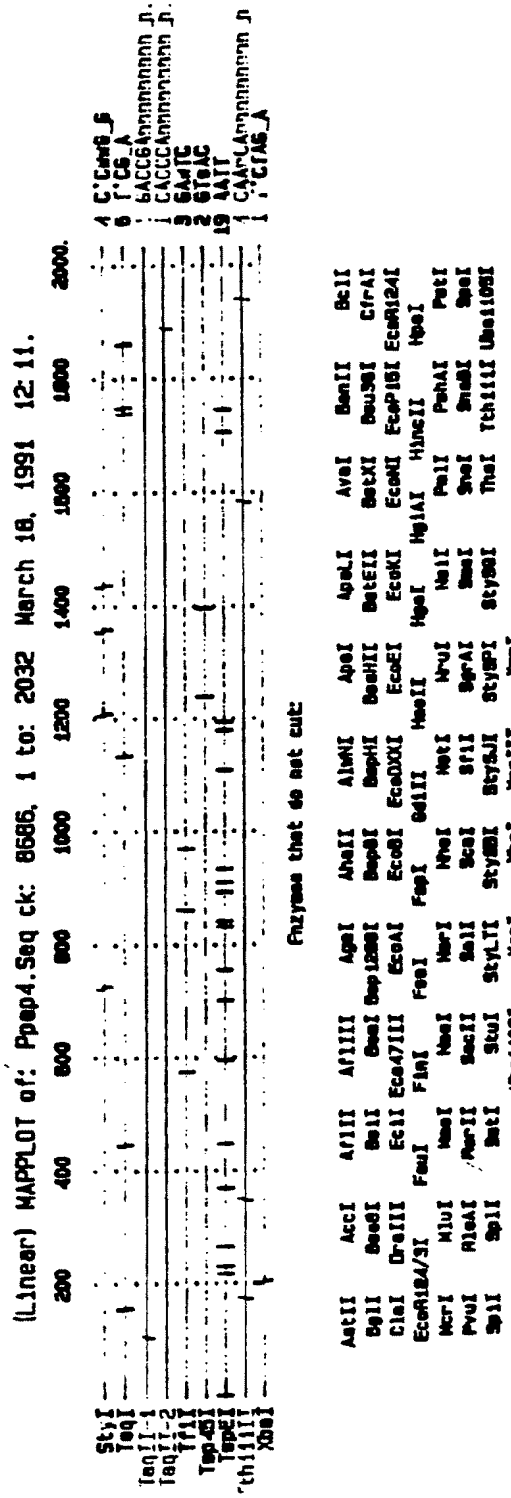

(Linear) MAPPLOT of: Ppep4.Seq ck: 8686, 1 to: 2032 March 18, 1991 12:11.
200
400
600
800
1000
1200
1400
1600
1800
2000.
StyI
TaqI
TaqII-1
TaqII-2
TfiI
Tsp45I
TspEI
thi111I
XbaI
4 C'CanG_B
6 F'CG_A
1 bACCGAnnnnnnnnn_B.
1 CACCCAnnnnnnnnn_B.
5 GAATC
2 GTGAC
19 AATT
1 CAAFCAnnnnnnnnn_B
1 F'CFAG_A
Enzymes that do not cut:
AatII AccI AflII AflIII AgeI AhaII AlwNI ApoI ApaLI AvaI BanII BclI
BglI BsaBI BsiI BsmI BsrI1208I BspEI BspHI BssHII BstEII BstXI Bsu36I ClaI
ClaI DraIII EclI Eco47III EcoAI EcoBI EcoDXXI EcoEI EcoKI EcoNI EcoP15I EcoR124I
EcoR124/3I FauI FlaI FseI FspI G411I HaeII HgaI HgiAI HincII HpaI
KpnI KluI NaeI NarI NheI NotI NruI NsiI PaeI PshAI PstI
PvuI RleAI SacII SalI ScaI SfiI SgrAI SmaI SnaBI SpeI
SphI SplI StuI StyLTI StyD4I StyI4I StySPI StyX1I ThaI Tth111I Ubs1105I
Ubs1104I XcaI XhoI XmaIII XmnI

FIG 1-3

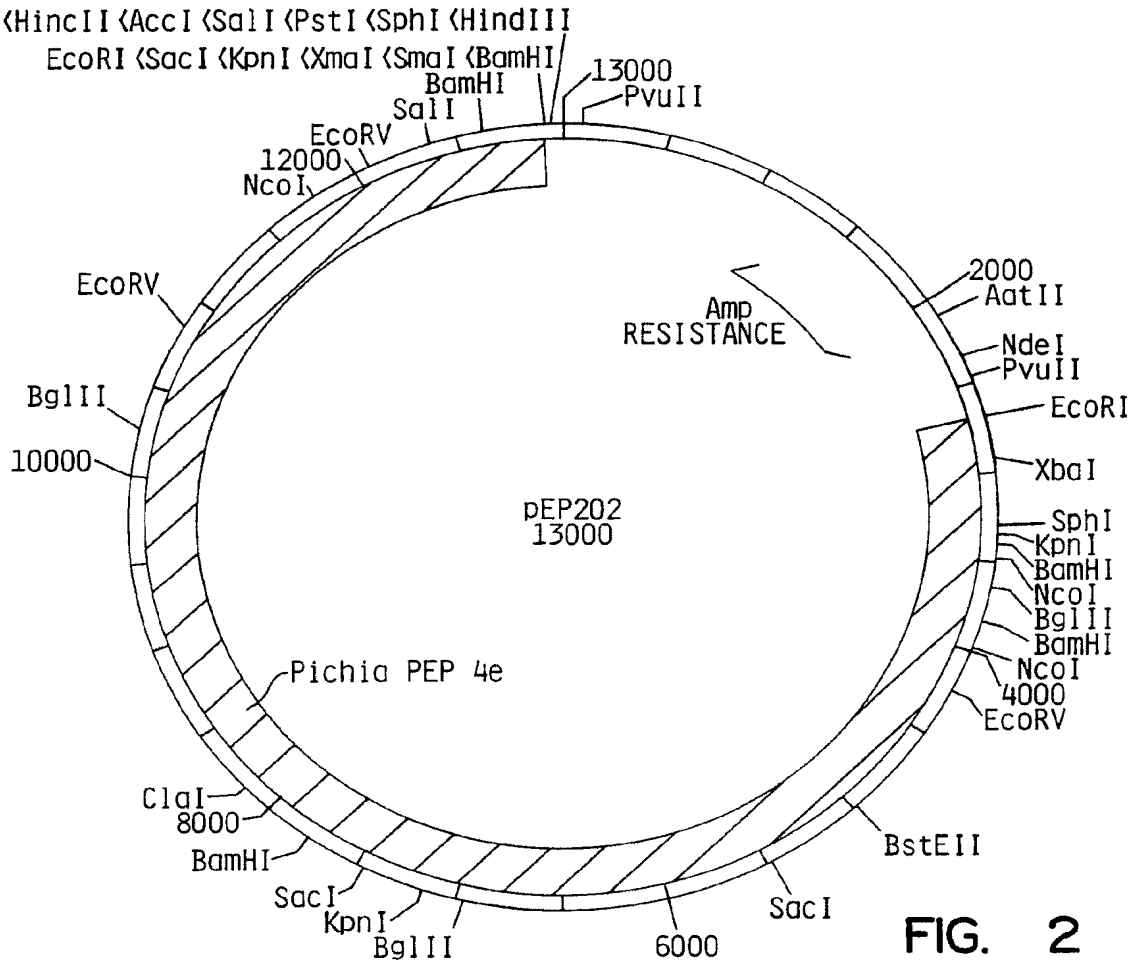
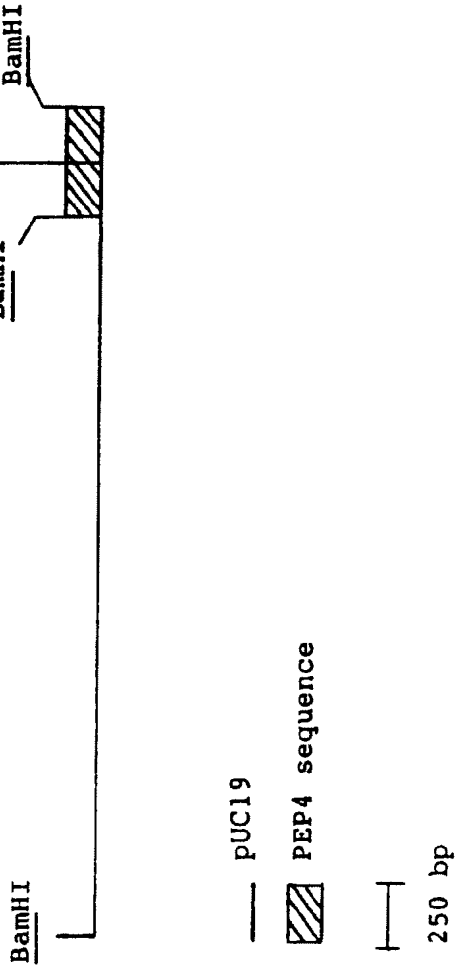

XbaI HincII AccI SalI PstI SphI HindIII
EcoRI SacI KpnI XmaI SmaI BamHI
BamHI
SalI
EcoRV
12000
NcoI
13000
PvuII
2000
AatII
NdeI
PvuII
EcoRI
XbaI
SphI
KpnI
BamHI
NcoI
BglII
BamHI
NcoI
4000
EcoRV
BstEII
SacI
6000
BglII
KpnI
SacI
BamHI
8000
ClaI
Pichia PEP 4e
10000
BglII
EcoRV
Amp
RESISTANCE
pEP202
13000

FIG. 2

FIGURE 3

Linear Restriction Map of pEP205



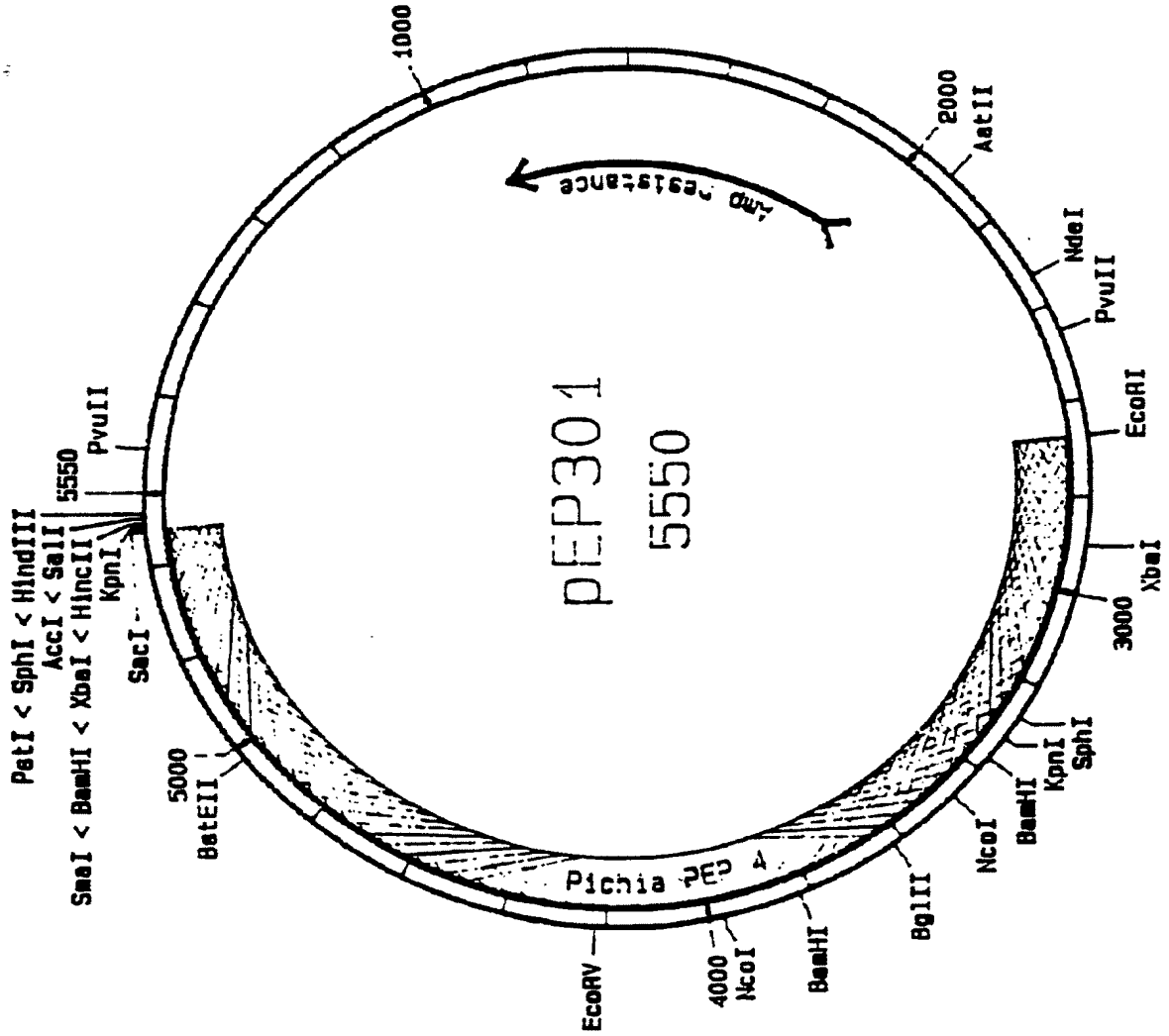

pEP301
5550
Amp resistance
Pichia PEP 4
1000
2000
AatII
NdeI
PvuII
EcoRI
XbaI
3000
SphI
KpnI
BamHI
NcoI
BglII
BamHI
4000
NcoI
EcoRV
5000
BstEII
SacI
KpnI
AccI < SalI
SmaI < BamHI < XbaI < HincII
PstI < SphI < HindIII
5550
PvuII

Fig 4

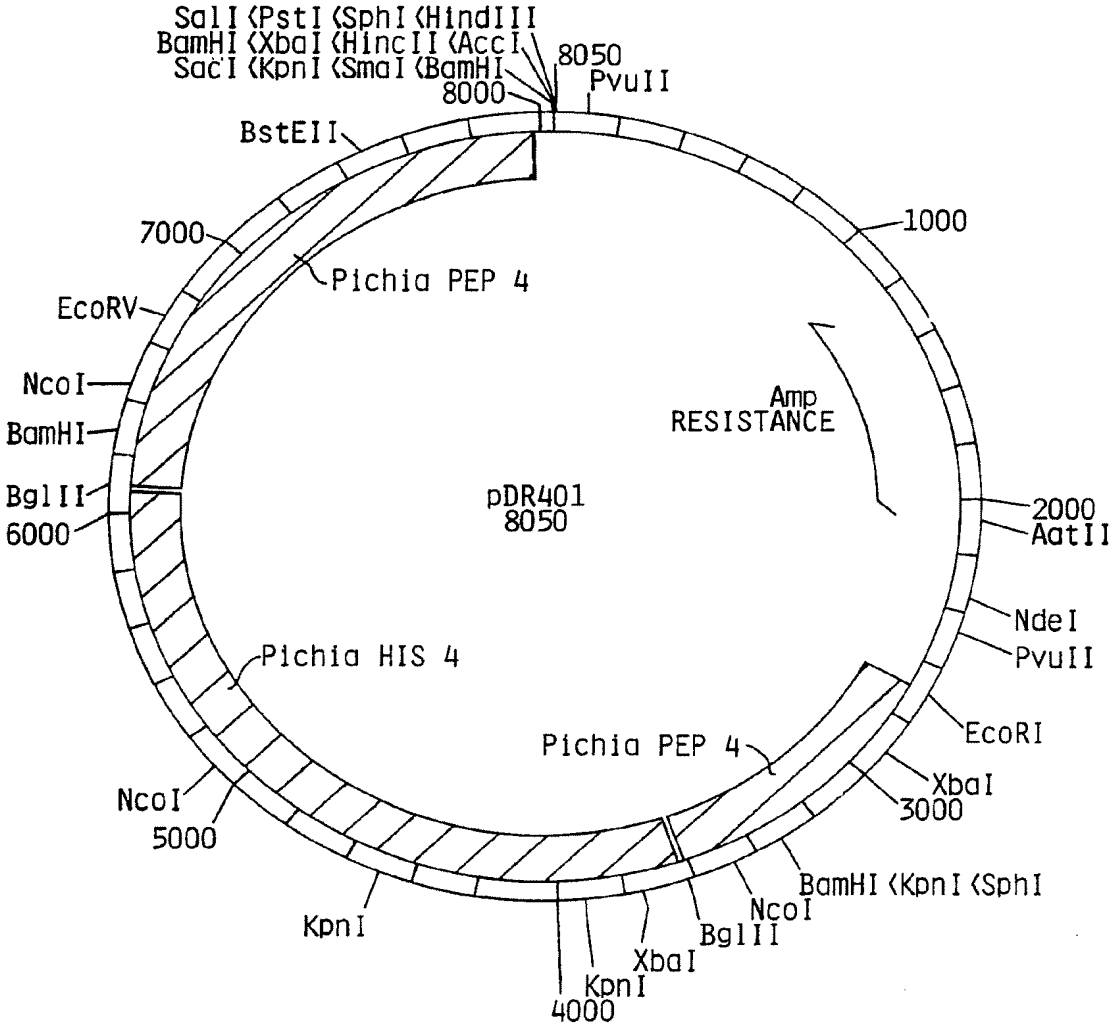

SalI (PstI (SphI (HindIII
BamHI (XbaI (HincII (AccI
SacI (KpnI (SmaI (BamHI
8050
PvuII
8000
BstEII
1000
7000
Pichia PEP 4
EcoRV
NcoI
BamHI
Amp
RESISTANCE
BglII
6000
pDR401
8050
2000
AatII
NdeI
PvuII
Pichia HIS 4
EcoRI
Pichia PEP 4
XbaI
3000
NcoI
5000
BamHI (KpnI (SphI
NcoI
KpnI
BglII
XbaI
KpnI
4000

FIG. 5

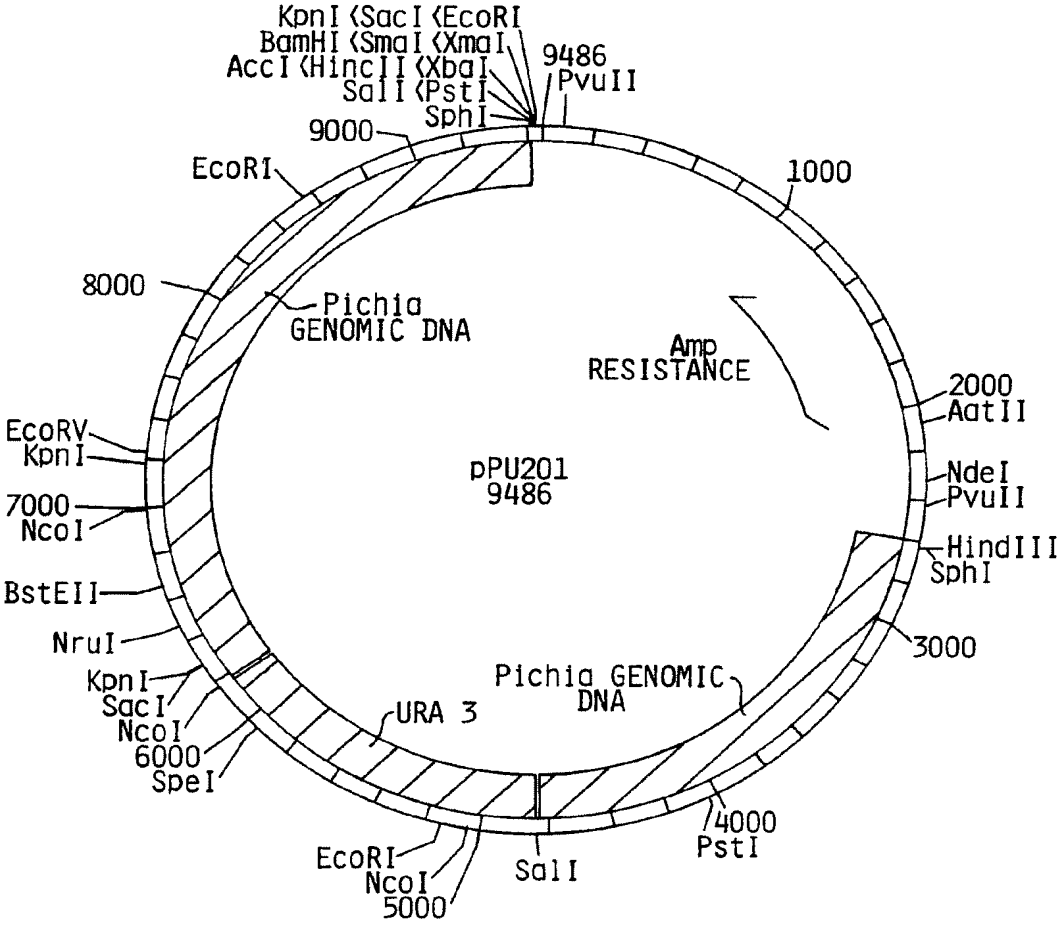
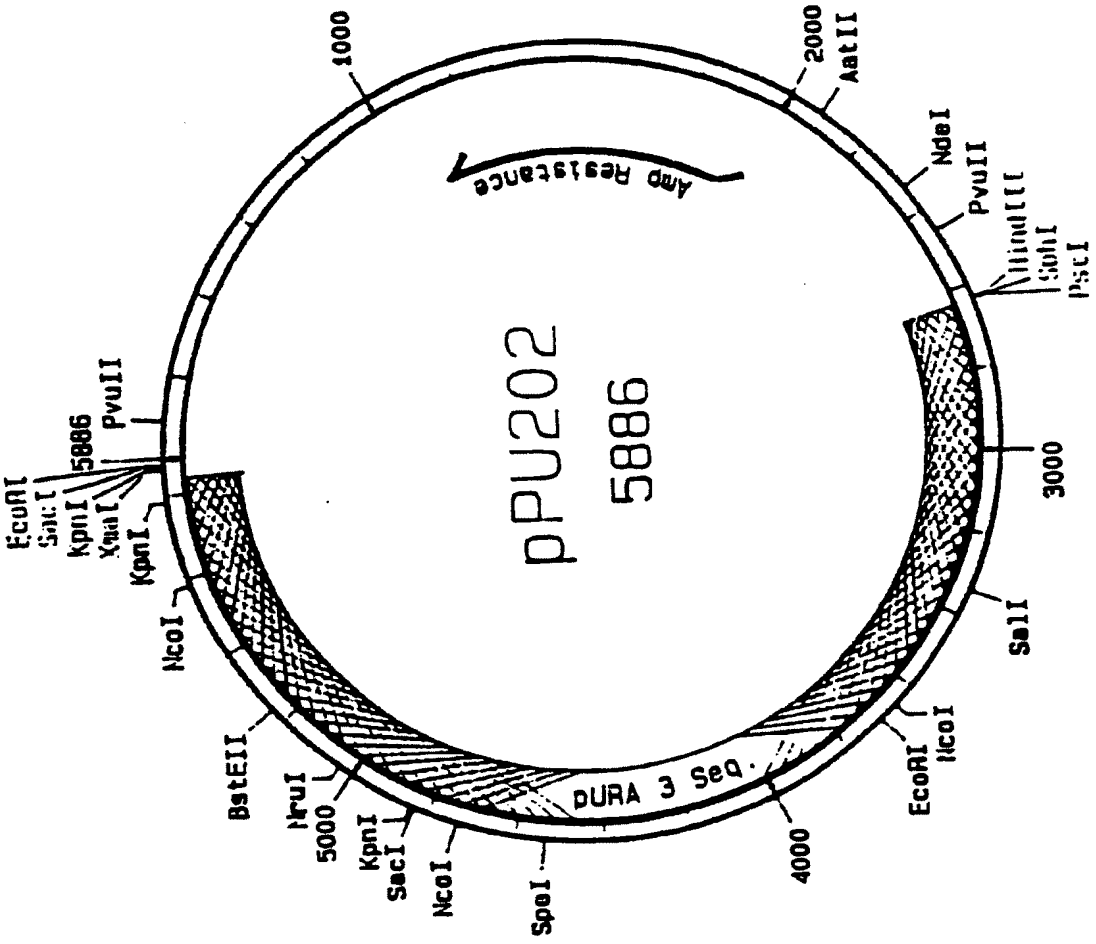

KpnI (SacI (EcoRI
BamHI (SmaI (XmaI
AccI (HincII (XbaI
SalI (PstI
SphI
9486
PvuII
9000
EcoRI
1000
8000
Pichia
GENOMIC DNA
Amp
RESISTANCE
2000
AatII
EcoRV
KpnI
pPU201
9486
NdeI
PvuII
7000
NcoI
HindIII
SphI
BstEII
NruI
3000
KpnI
SacI
NcoI
6000
SpeI
Pichia GENOMIC
DNA
URA 3
4000
PstI
EcoRI
NcoI
5000
SalI

FIG. 6

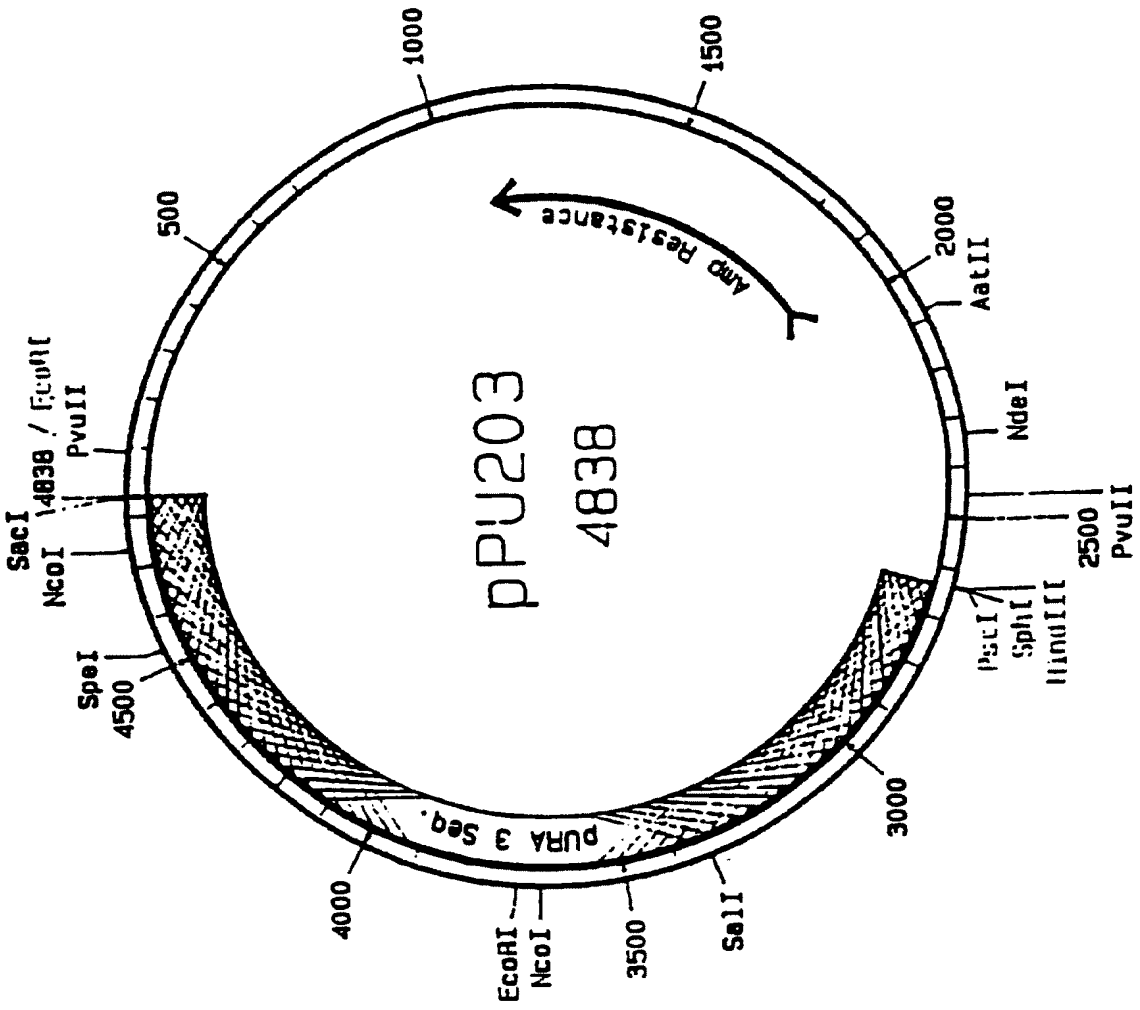
FIG 7



PLASMIDMAP of: pPU202.Seq check: 5464 from: 1 to: 5886

Howard April 4, 1990 08:59

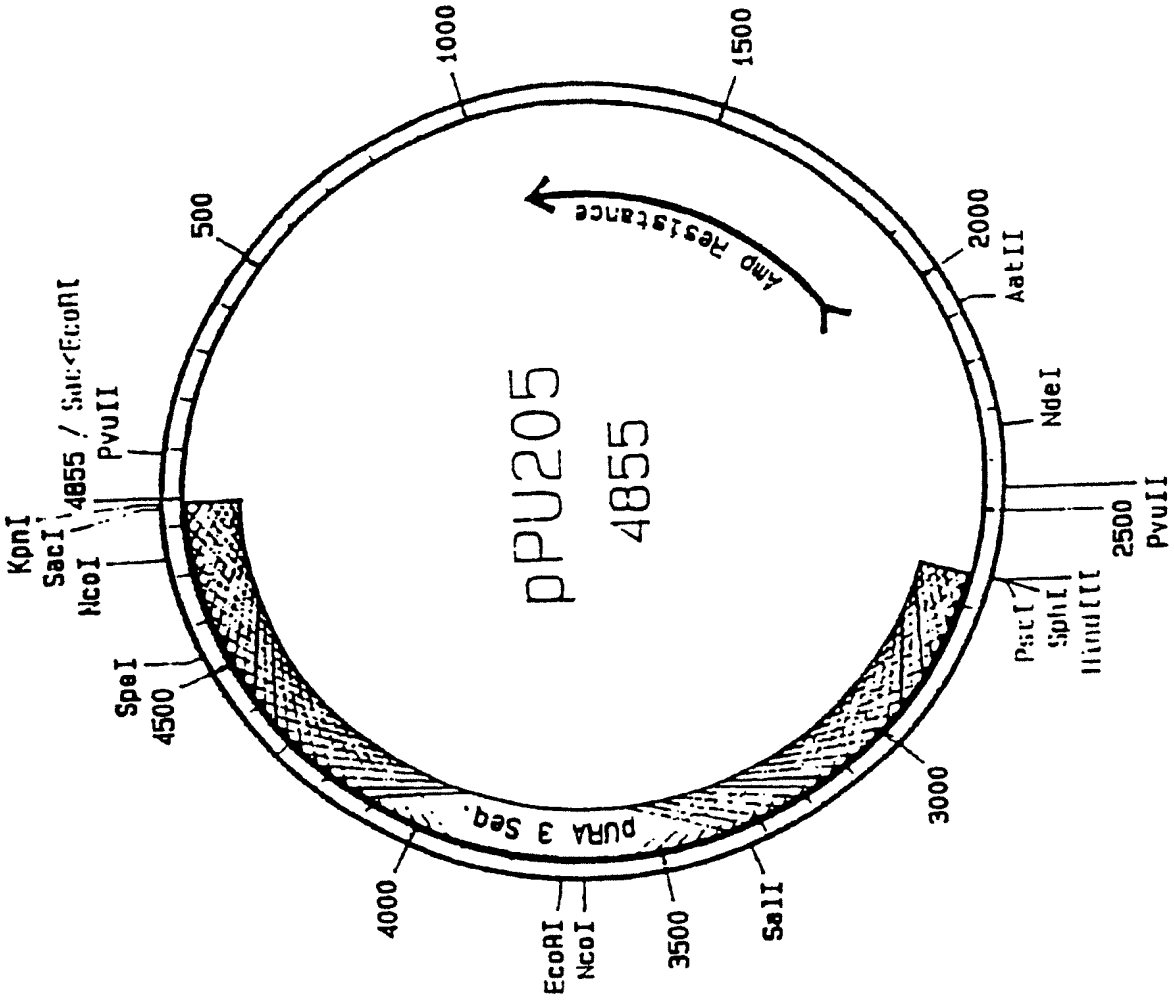
FIG 8



PLASMIDMAP of: pPU203.Seq check: 5464 from: 1 to: 4838

Howard April 4, 1990 10:19

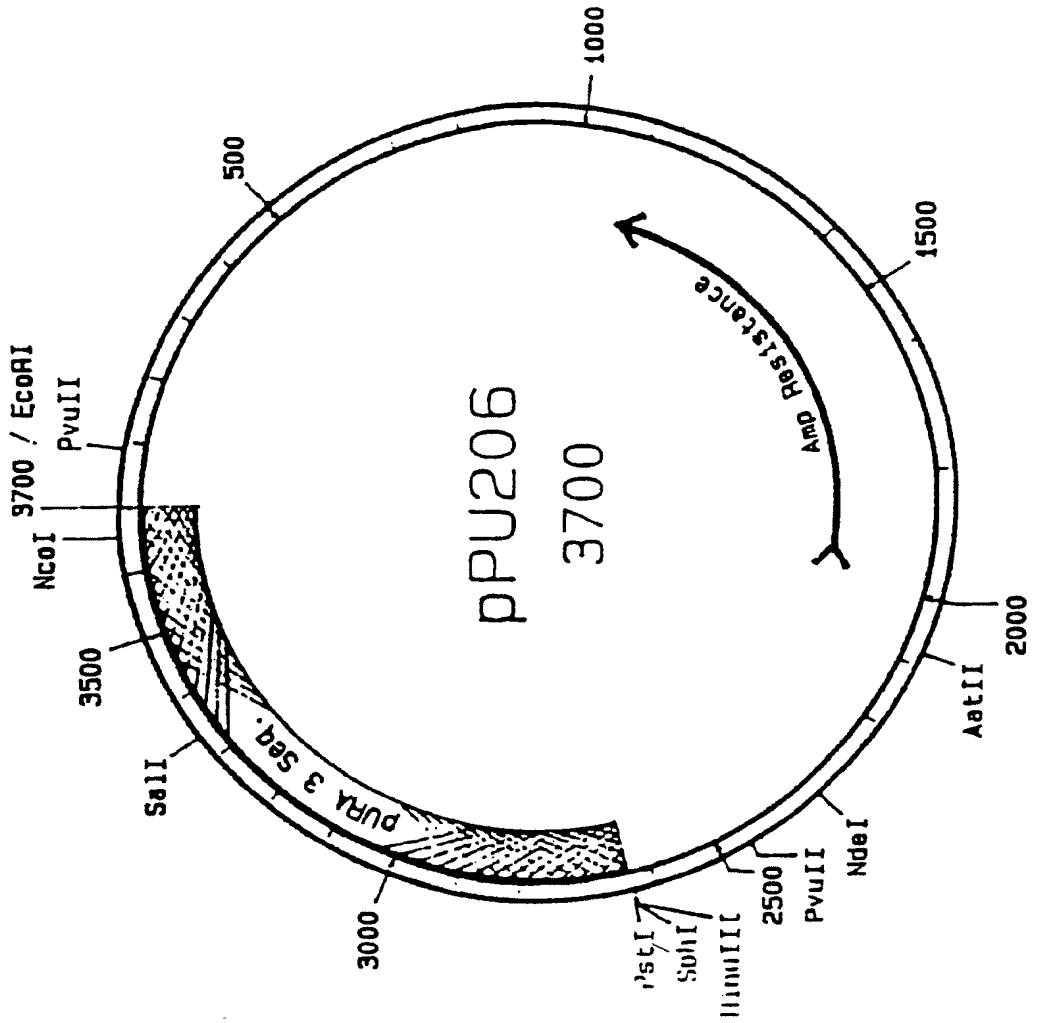
FIG 9



PLASMIDMAP of: pPU205.Seq check: 5464 from: 1 to: 4855

Howard April 4, 1990 10:33

FIG 1D



PLASMIDMAP of: pPU206.Seq check: 5464 from: 1 to: 3700

Howard April 4, 1990 10:39

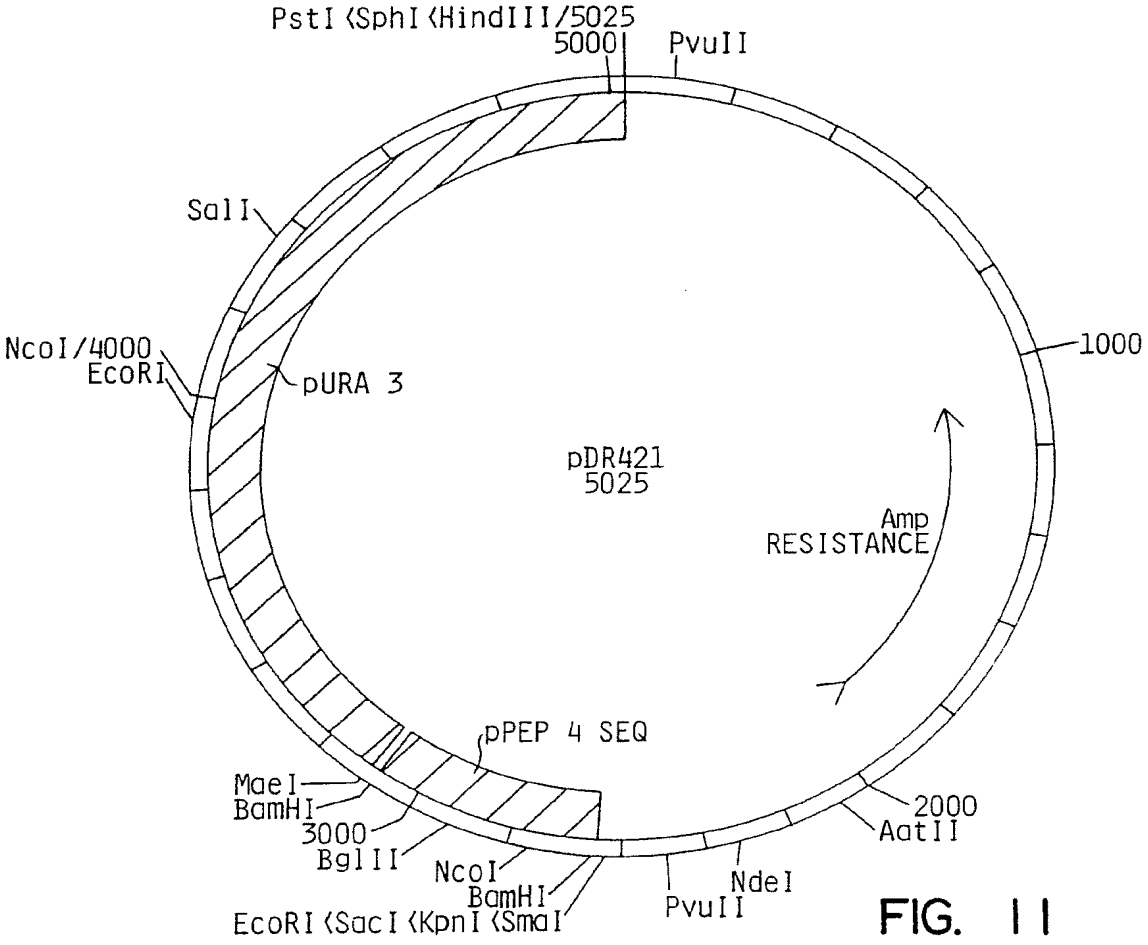

PstI (SphI (HindIII/5025
5000
PvuII
SalI
NcoI/4000
EcoRI
pURA 3
1000
pDR421
5025
Amp
RESISTANCE
pPEP 4 SEQ
MaeI
BamHI
3000
BglII
NcoI
BamHI
PvuII
NdeI
2000
AatII
EcoRI (SacI (KpnI (SmaI

FIG. 11

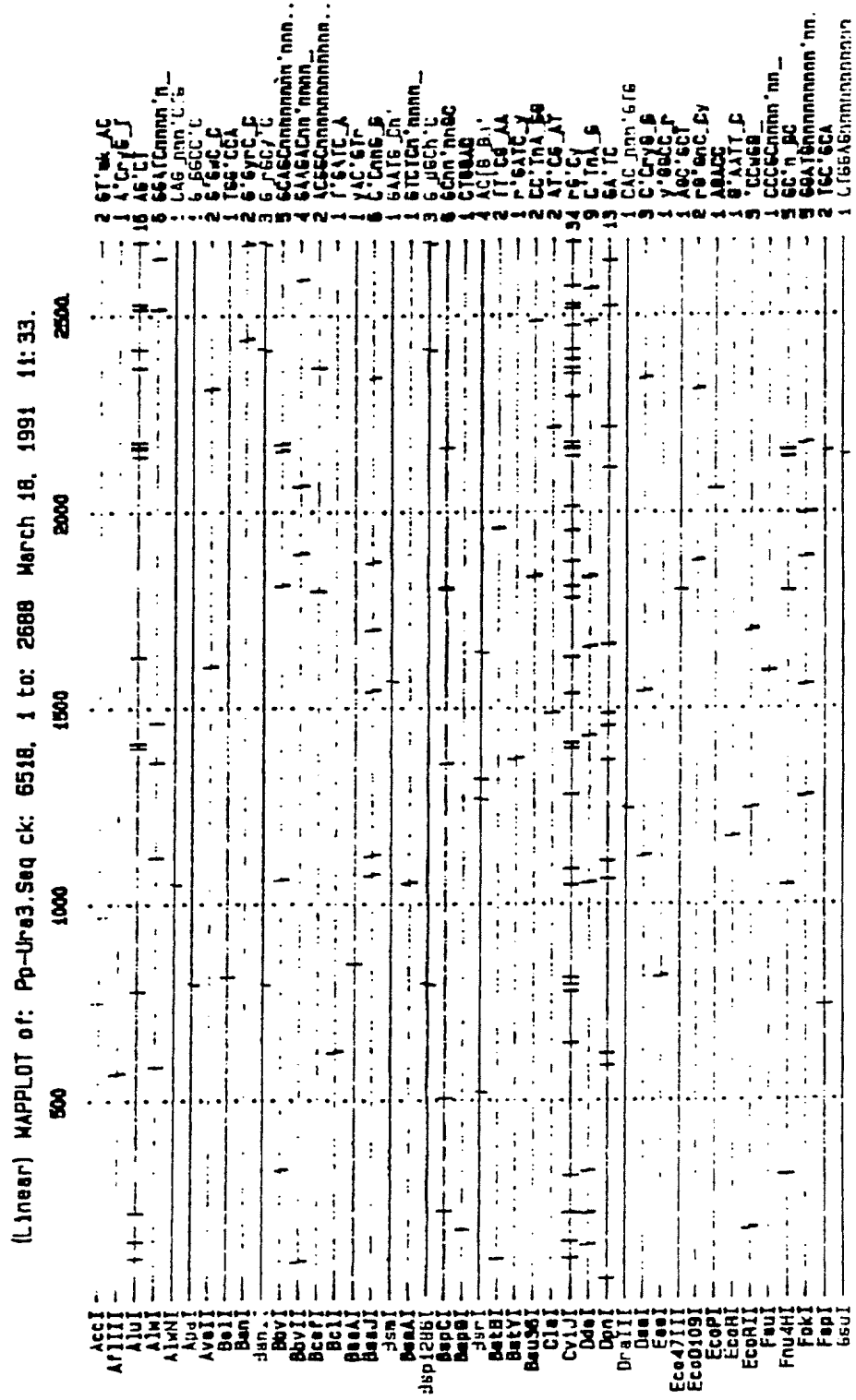

(Linear) MAPPLOT of: Pp-Ura3.Seq ck: 6518, 1 to: 2688 March 18, 1991 11:33.
500
1000
1500
2000
2500
AccI
ApaLI
AluI
AlwI
AlwNI
ApaI
AvaII
BclI
BanI
BbvI
BbvII
BcefI
BclI
BseAI
BseJI
BsmI
BseAI
Bsp120I
BspCI
BspEI
BsrI
BstBI
BstYI
Bsu36I
ClaI
CviJI
DdeI
DpnI
DraIII
DseI
EaeI
Eco47III
Eco0109I
EcoPI
EcoRI
EcoRII
FauI
Fnu4HI
FokI
FspI
HaeI

FIG. 12-1

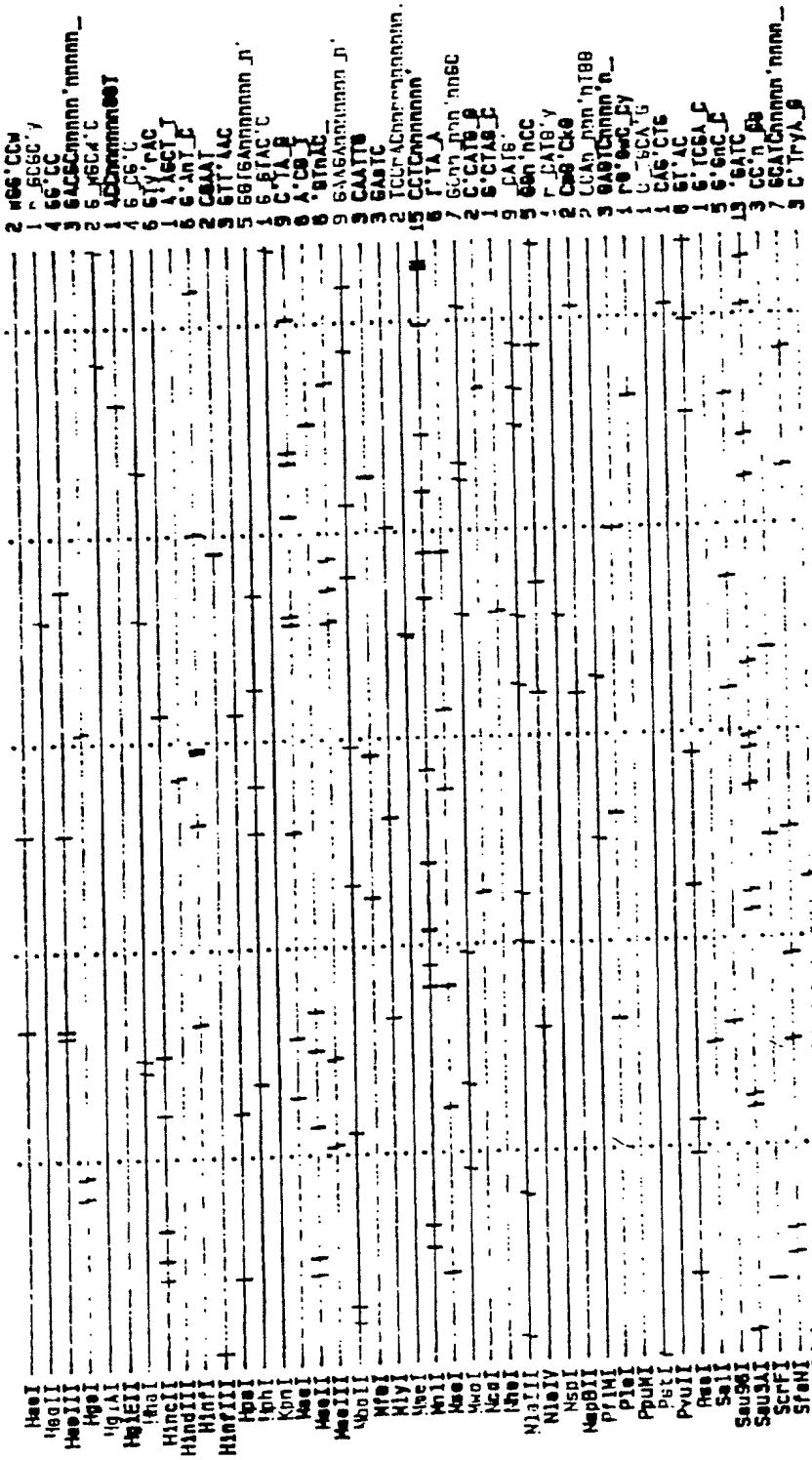

(Linear) MAPPLOT of: Pp-Ure3.Seq ck: 6518, 1 to: 2688 March 18, 1991 11:34.
500
1000
1500
2000
2500.
HaeI
HgaI
HaeIII
HgiAI
HphI
HincII
HindIII
HinfI
HinfIII
HpaI
HpaII
KpnI
MaeI
MaeII
MaeIII
MboII
MfeI
MlyI
MseI
MslI
NaeI
NarI
NcoI
NheI
NlaIII
NlaIV
NspI
NspBII
PflMI
PleI
PpuMI
PstI
PvuII
RsaI
SalI
Sau96I
Sau3AI
ScrFI
SfaNI
SfoI

FIG. 12-2

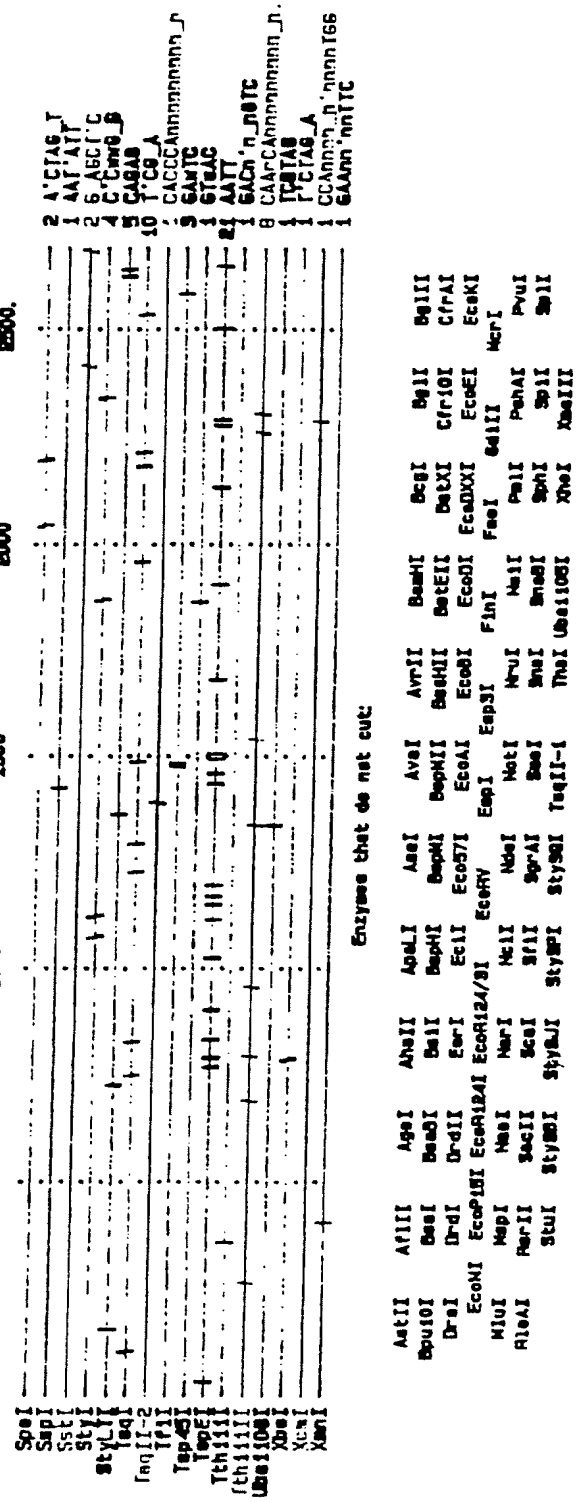

(Linear) MAPPLOT of: Pp-Ura3.Seq ck: 6518, 1 to: 2688 March 18, 1991 11:45.
500
1000
1500
2000
2500.
SpeI
SapI
SstI
StyI
TaqI
TaqII-2
TfiI
Tsp45I
TspEI
Tth111I
Uba1108I
XbaI
XcmI
XmnI
Enzymes that do not cut:
AatII AflII AgeI AhaII ApaLI AseI AvaI AvrII BamHI BcgI BglI BglII
BpuI0I BsaI BseDI BsaHI BspHI BspMII BspNII BstEII BstXI Cfr10I CfrAI
DraI DrdI DrdII EarI EclI Eco57I EcoAI EcoBI EcoDI EcoDXXI EcoEI EcoKI
EcoNI EcoP15I EcoR124I EcoR124/3I EcoRV EspI Esp3I FinI FseI GdiII NcrI
KluI MapI NaeI NarI NciI NdeI NotI NruI NsiI PaiI PshAI PvuI
RleAI RsrII SacII ScaI SfiI SgrAI SmaI SnaI SnoBI SphI SplI SpiI
StuI StyD4I StyI0I StyHJI StyMPI StySBI TaqII-1 ThaI Uba1108I XhoI XmaIII

FIG. 12-3

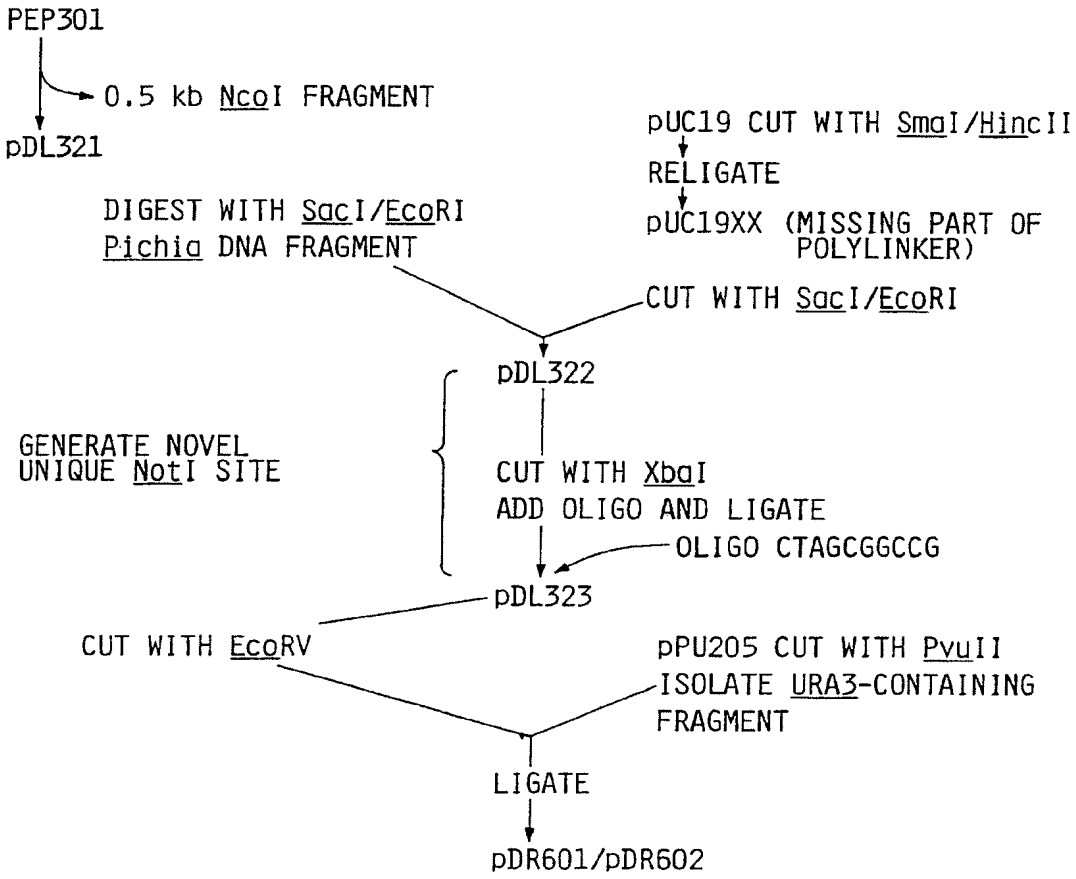

PEP301
0.5 kb NcoI FRAGMENT
pDL321
DIGEST WITH SacI/EcoRI
Pichia DNA FRAGMENT
pUC19 CUT WITH SmaI/HincII
RELIGATE
pUC19XX (MISSING PART OF POLYLINKER)
CUT WITH SacI/EcoRI
pDL322
GENERATE NOVEL UNIQUE NotI SITE
CUT WITH XbaI
ADD OLIGO AND LIGATE
OLIGO CTAGCGGCCG
pDL323
CUT WITH EcoRV
pPU205 CUT WITH PvuII
ISOLATE URA3-CONTAINING FRAGMENT
LIGATE
pDR601/pDR602

FIG. 13

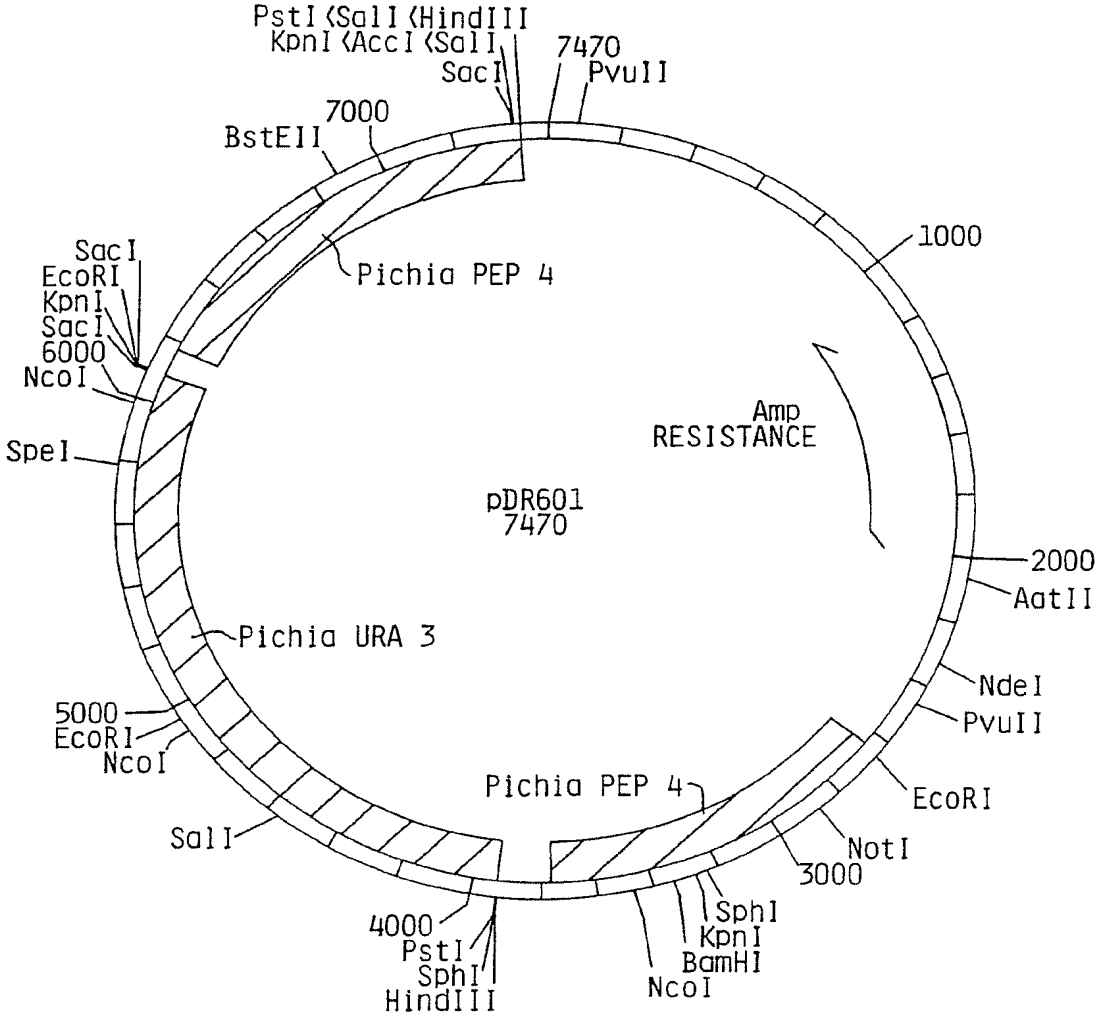

PstI (SalI (HindIII
KpnI (AccI (SalI
SacI
7470
PvuII
7000
BstEII
1000
Pichia PEP 4
SacI
EcoRI
KpnI
SacI
6000
NcoI
SpeI
Amp
RESISTANCE
pDR601
7470
2000
AatII
Pichia URA 3
NdeI
PvuII
5000
EcoRI
NcoI
Pichia PEP 4
EcoRI
SalI
NotI
3000
4000
PstI
SphI
HindIII
SphI
KpnI
BamHI
NcoI

FIG. 14

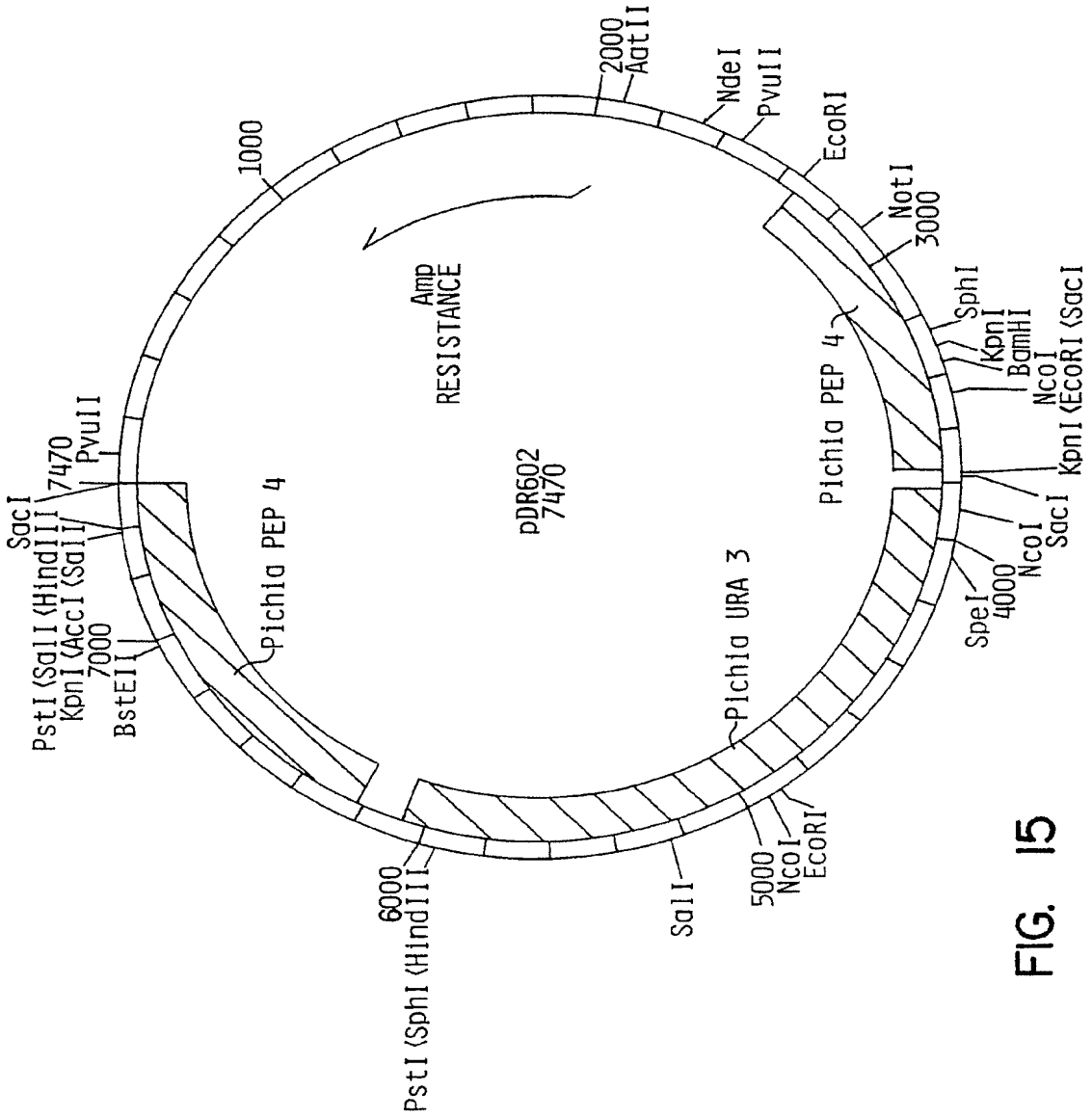

2000
AatII
NdeI
PvuII
EcoRI
NotI
3000
SphI
KpnI
BamHI
NcoI
(EcoRI (SacI
KpnI
SacI
NcoI
4000
SpeI
Pichia PEP 4
Pichia URA 3
SalI
5000
NcoI
EcoRI
6000
PstI (SphI (HindIII
pDR602
7470
Amp
RESISTANCE
1000
Pichia PEP 4
7000
BsteII
PstI (SalI (HindIII
KpnI (AccI (SalI
SacI
7470
PvuII

FIG. 15

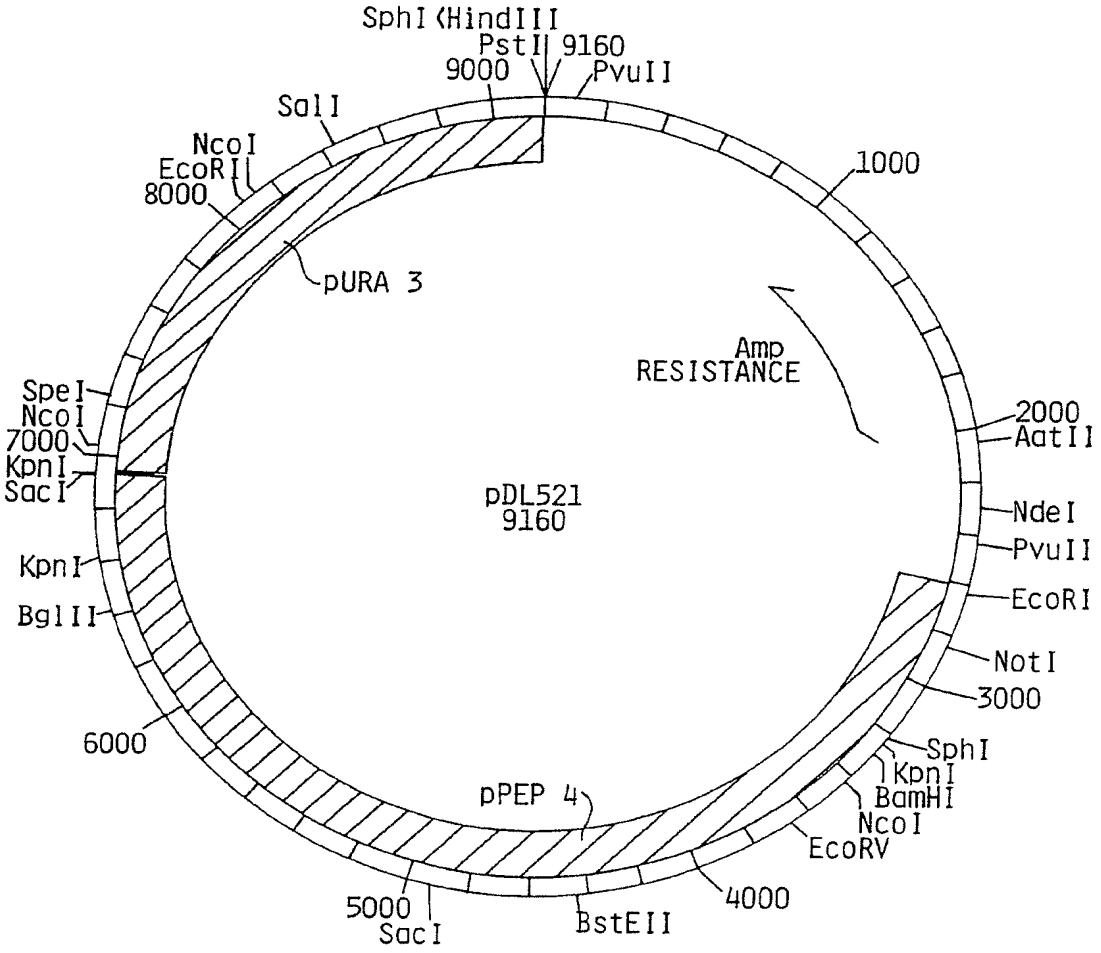
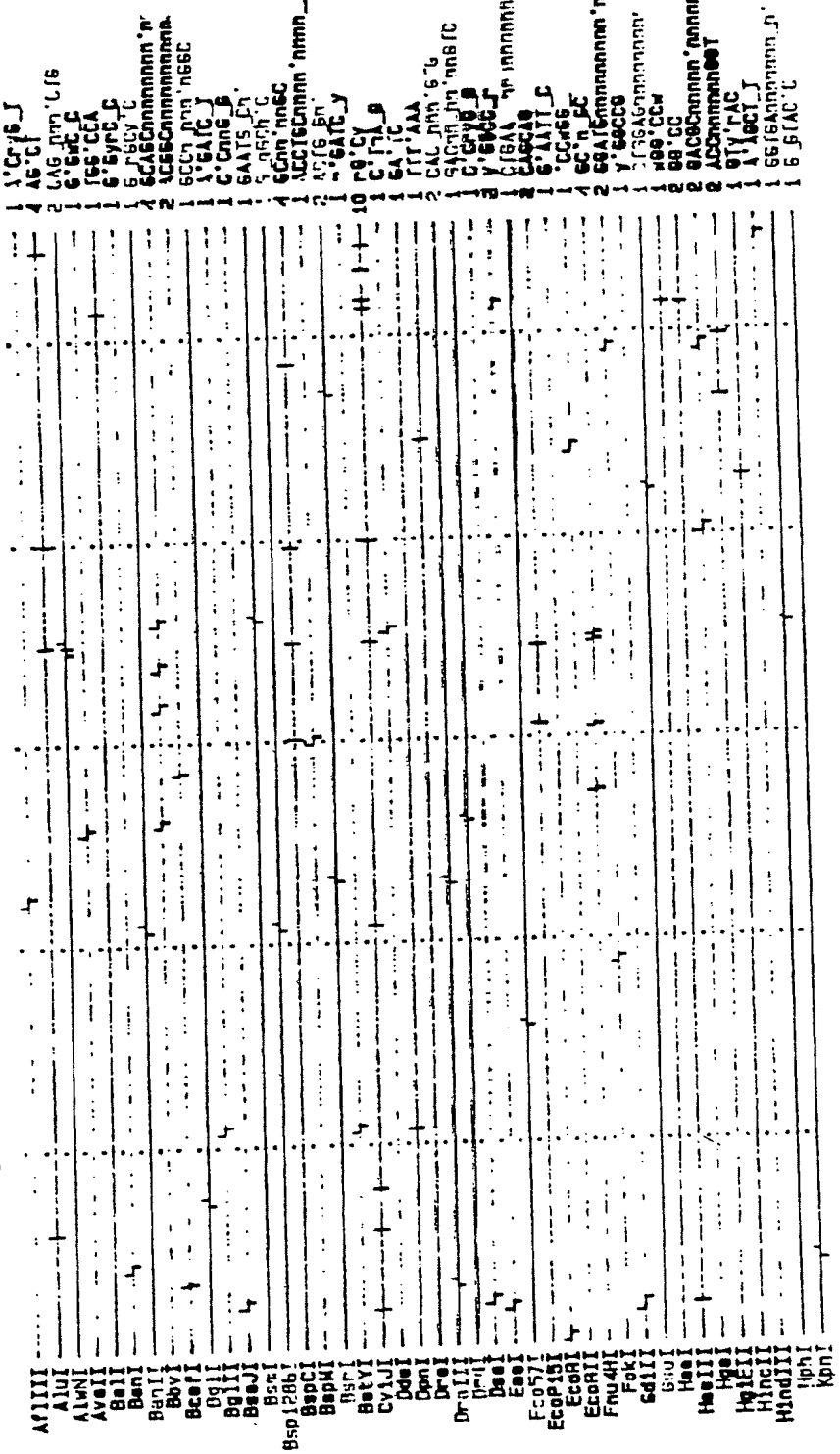
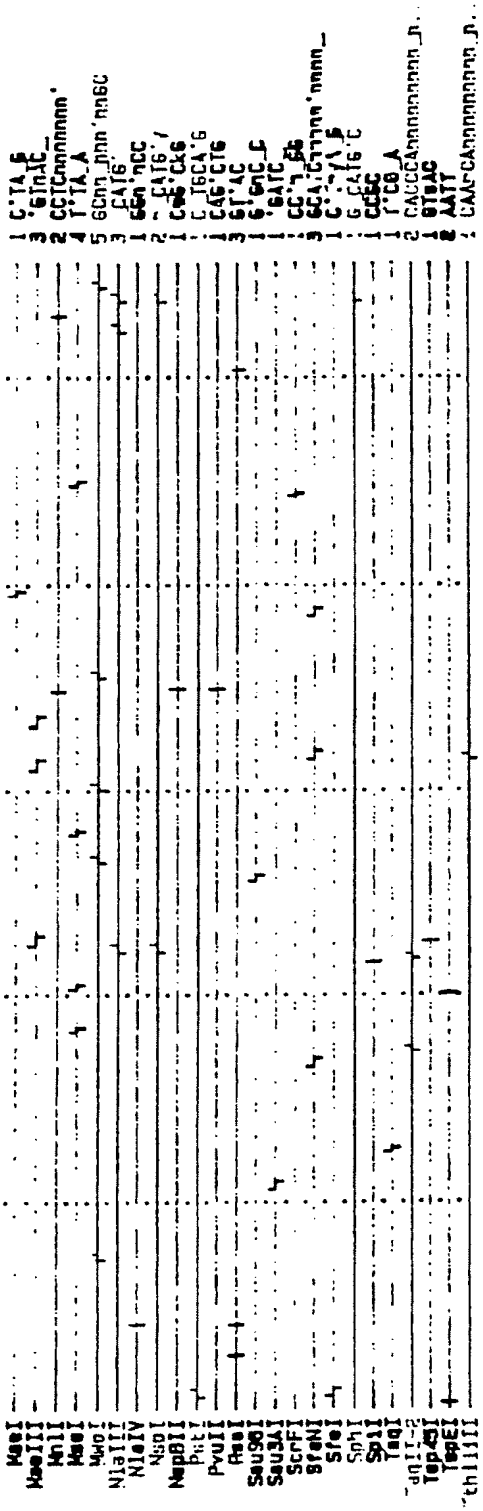

SphI (HindIII
PstI 9160
9000
PvuII
SalI
NcoI
EcoRI
8000
1000
pURA 3
Amp
RESISTANCE
SpeI
NcoI
7000
KpnI
SacI
2000
AatII
pDL521
9160
NdeI
PvuII
KpnI
EcoRI
BglII
NotI
3000
6000
SphI
KpnI
BamHI
NcoI
EcoRV
pPEP 4
5000
SacI
BstEII
4000

FIG. 16


(Linear) MAPPLOT of: Pp-Prb1.Seq ck: 7710. 1 to: 555 March 18, 1991 12:28.
100
200
300
400
500
AflIII
AluI
AlwNI
AvaII
BalI
BanI
BanII
BbvI
BcefI
BglI
BglII
BseJI
BsmI
Bsp120bI
BspCI
BspHI
BsrI
BstYI
CviJI
DdeI
DpnI
DraI
DraIII
DrdI
EaeI
EcoRI
EcoRII
Fnu4HI
FokI
GdiII
HaeI
HaeIII
HgaI
HgiEII
HincII
HindIII
NphI
XmnI

19-1


(Linear) NAPPLOT of: Pp-Prb1.Seq ck: 7710. 1 to: 555 March 18, 1991 12:28.
100
200
300
400
500.
Enzymes that do not cut:

17-2

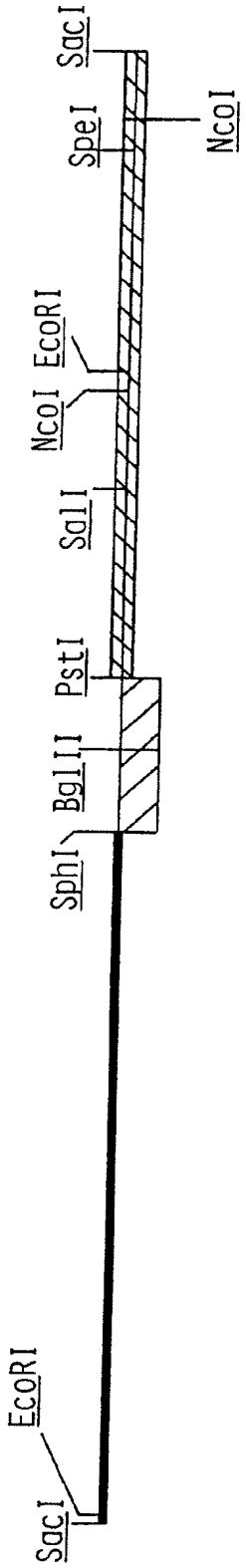

SacI
SpeI
NcoI
NcoI
EcoRI
SalI
PstI
BglII
SphI
EcoRI
SacI
PRB-1 GENE SEQUENCE
URA3 GENE SEQUENCE
pUC19

FIG. 18

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/02521

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/81; C12N15/57; C12N15/53; C12N15/60
C12N1/19; C07K13/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	EP,A,0 336 056 (M & D RESEARCH CO) 11 October 1989 see page 1, last paragraph - page 2; claim 2	1
A	CHEMICAL ABSTRACTS, vol. 106, 1987, Columbus, Ohio, US; abstract no. 48594, G. NELSON AND T. YOUNG: 'Yeast extracellular proteolytic enzymes for chill-proofing beer' page 505 ;column L ; see abstract & J. INST. BREW. vol. 96, no. 6, 1986, pages 599 - 603;	1

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 AUGUST 1992	20. 08. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	VAN DER SCHAAL C.A.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 390 676 (TRANSGENE S.A.) 3 October 1990 see the whole document	8,9,11, 12,14, 16,17, 19,22, 23,26, 28-30
A	BIOTECHNOLOGY vol. 7, no. 2, February 1989, NEW YORK US pages 160 - 164; M. DIGAN ET AL: 'Continuous production of a novel lysozyme via secretion from the yeast, Pichia pastoris' see the whole document	32
A	WO,A,9 009 449 (HENKEL RESEARCH CORPORATION) 23 August 1990 see abstract; claims	22,31, 32,34, 39-48
A	GENE. vol. 29, 1984, AMSTERDAM NL pages 113 - 124; M. ROSE: 'Structure and function of the yeast URA3 gene: expression in Escherichia coli' see the whole document	39
A	MOLECULAR AND CELLULAR BIOLOGY vol. 6, no. 7, July 1986, WASHINGTON, US pages 2490 - 2499; G. AMMERER ET AL: 'PEP4 gene of Saccharomyces cerevisiae encodes Proteinase A, a vacuolar enzyme required for processing of vacuolar precursors' see abstract; figure 2	2,5,10, 18,20, 24,29, 31,36,47

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9202521 SA 59408

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 11/08/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0336056	11-10-89	JP-A-	1191683	01-08-89
		JP-A-	2049585	19-02-90
		US-A-	5053333	01-10-91
EP-A-0390676	03-10-90	FR-A-	2645175	05-10-90
		CA-A-	2013240	30-09-90
		JP-A-	3072868	28-03-91
WO-A-9009449	23-08-90	EP-A-	0457852	27-11-91